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STABILITY OF RAT TISSUE LIPIDS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
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by

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ABSTRACT

The stability of lipids and the effect of vitamin E deficiency on lipid stability in several rat tissues were examined. All normal rat tissues exhibited some evidence of lipid peroxidation in vivo and most of them showed a susceptibility to peroxidation in vitro. However, this susceptibility to peroxidation was much greater in tissues from rats kept on vitamin E deficient diets.

In vitamin E deficient animals, the tissue response--in order of decreasing activity--with respect to lipid susceptibility to peroxidation, was adrenal, heart, lung and liver. With the exception of brain, the polyunsaturated fatty acid content of all tissues from vitamin E deficient rats was lower than that of the corresponding tissues from control animals.

Brain exhibited relatively high levels of peroxidation products in vivo. In this tissue, neither the level nor the vulnerability to peroxidation of the polyunsaturated fatty acids appeared to be affected by the diet on which the experimental animal was maintained.

At the sub-cellular level, the microsomal-supernatant fraction from normal brain and heart was found to have higher levels of lipid peroxidation products and greater susceptibility to peroxidation in vitro than did the mitochondria. Brain microsomal-supernatant fractions from normal and the vitamin E deficient rats did not show any significant differences either in the amount of peroxidation products, or in the polyunsaturated fatty acid content. Heart microsomal-supernatant fractions from vitamin E deficient rats, on the other hand, showed a much higher susceptibility to peroxidation in vitro, and a greater loss of polyunsaturated fatty acids on aerobic incubation than did corresponding preparations from normal animals. Feeding corn oil to the vitamin E deficient animals did not restore their tissue polyunsaturated fatty acids levels to normal.

Studies relating to the mechanism of lipid peroxidation confirmed the findings that under physiological conditions one of the main end-products of lipid peroxidation (estimated by the thiobarbituric acid

test) is malonaldehyde. Spectrophotometric studies suggested the presence of another compound which formed a chromogen with thiobarbituric acid having an absorption peak at 450 mμ. Subsequent studies indicated that this compound might be a precursor of malonaldehyde.

Heavy metal ions--ferrous, ferric and vanadyl--and ascorbic acid accelerated lipid peroxidation in vitro in brain homogenates while cobaltic, cupric and manganous ions as well as certain antioxidants inhibited the reaction. Heating brain homogenates accelerated lipid peroxidation but did not block further lipid peroxidation. It is therefore suggested that this is a metal catalyzed, non-enzymic process in vitro and that the in vivo situation might be somewhat similar.

Studies with lipid extracts from tissue homogenates suggested that the events occurring during aerobic incubation are concerned primarily with the breakdown of preformed peroxidic compounds rather than with further oxidation of polyunsaturated fatty acids.

It has been shown that although the thiobarbituric acid test is not quantitative, it is highly sensitive and by controlling rigorously the conditions with respect to both the tissue pretreatment and the test itself, it provides a reasonably good estimate of lipid peroxidation.

In the newborn rat, brain tissue was shown to contain a high level of lipid peroxidation products. The level of these products decreased progressively with increasing age of the animal until it reached a relatively constant value at 10-15 days postpartum. The level of polyunsaturated fatty acids, on the other hand, increased from birth until about the 25th day of age. It appears that during maturation, some factor or process is gradually introduced into, or generated within, the brain, which retards the tendency of the polyunsaturated fatty acids to undergo oxidation in situ.

The developing rat heart showed a behavior somewhat similar to that of brain with respect to the tendency of its polyunsaturated fatty acids to peroxidize. The microsomal-supernatant fractions from both these tissues exhibited a similar pattern during early development.

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ABBREVIATIONS

DPPD	-	N, N'-diphenyl-p-phenylenediamine
EDTA	-	Ethylenediaminetetraacetate
EFA	-	Essential Fatty Acids
Hb	-	Hemoglobin
MA	-	Malonaldehyde
MA-TBA	-	Malonaldehyde-thiobarbituric acid coloured complex
MB	-	Methylene-blue
Mb	-	Myoglobin
PFA	-	Polyunsaturated fatty acids
RBC	-	Red blood cell (erythrocyte)
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
Tris	-	Trishydroxy methyl amino methane
UFA	-	Unsaturated fatty acids

I. INTRODUCTION

The purpose of this study was to investigate the stability of polyunsaturated fatty acids (PFA), in various rat tissues, especially brain and heart. The PFA, in common with many other unsaturated substances, undergo spontaneous oxidation by atmospheric oxygen. The result of prolonged oxidation of these lipids is the development of rancidity. Light, heat and certain impurities such as metals, accelerate the reaction. Many investigators have studied the mechanisms of the reactions involved in the development of rancidity, while others have tried to evaluate lipid stability; i.e. the resistance of fats to rancidification. Unsaturated fatty acids are the major substrates of the oxidative processes which lead to the development of rancidity. The initial step in the process is generally known as the peroxidation reaction, which involves the addition of oxygen at the carbon atom occupying the alpha position with respect to the double bond in the fatty acid chain, with the formation of a hydroperoxide.

The objectives of the investigations described here were as follows: (a) to determine whether differences exist in the rates of peroxidation of PFA in tissues from normal and vitamin E deficient rats; (b) to investigate peroxidation reaction at a sub-cellular level; (c) to investigate the end-products of the peroxidation of polyunsaturated fatty acids; (d) to study the effects of accelerators and inhibitors on the peroxidation reaction; and (e) to examine the accumulation of PFA and their susceptibility to peroxidation in the developing rat brain.

Dam and Granados in 1945 (1) reported that PFA tend to peroxidize in tissues of rats maintained on vitamin E deficient diets. However, Bernhard (2) in 1958 claimed that vitamin E had no effect on PFA levels in either rat brain or liver. Cole (3) and Kibrick (4) reported the presence of lipid peroxides in certain tissues of normal Golden hamster and rat, respectively, while Søndergaard, Prange and Dam (5) reported that these substances were absent from brain.

It was of interest therefore to determine whether lipid peroxides

exist in vivo, and if so, to learn something of how they are affected by vitamin E deficiency. The effect of peroxidation on the PFA content of the tissues was also investigated.

Most of the studies reported in the literature have been done in vitro, using manometric methods, which give a measure of the rate of the peroxidation reactions. In this study, an attempt was made to find out if a relationship exists between the in vivo PFA and peroxide levels in tissues from control and vitamin E deficient rats.

Tappel and associates (6,7) working with rabbit mitochondria, demonstrated that the functions of these cell particulates were affected by peroxidation, and that these aberrations were reducible by vitamin E. Ottolenghi in 1959 (8) reported similar findings. Microsomes contain twice as much PFA as mitochondria, on the basis of total nitrogen (Tappel(9) and Biran and Bartley (10)). It appeared worthwhile, therefore, to study peroxidation reactions in the different sub-cellular fractions.

As will be discussed in the following section, the initial steps of peroxidation are quite clear, whether the peroxidation is spontaneous or catalyzed by hematin or an enzyme(s). But the mechanism of hydroperoxide deterioration to give the large number of end-products is not so clear. Malonaldehyde is an end-product of peroxidation (11,12,13, 14). An attempt was made to isolate the chromogen(s) formed by the reaction of thiobarbituric acid (TBA) with the lipid peroxidation products, and to characterize these pigments by spectral and chromatographic methods. Certain substances other than malonaldehyde may react with TBA to form TBA-chromogens. However, these chromogens are produced under widely different conditions from those of lipid peroxidation and their absorption maxima are at wavelengths different from that of the MA-TBA chromogen (15,16,17).

Metallic ions have been shown to catalyze the oxidation of unsaturated fatty acids (Uri (18)). Influence of certain metallic ions and antioxidants, etc. on lipid peroxidation of the brain homogenates was examined in the present study. These observations were extended to investigations of the effects of metallic ions on the peroxidation of lipids in particulate fraction of cells. Recently, Kitabchi and associates (16) have also

reported the catalytic effect of metallic ions on lipid peroxidation at sub-cellular level.

A large proportion of the PFA in adult rat brain is associated with the phospholipid fraction (19,20,21). These PFA are quite susceptible to peroxidation in most animal tissues (22). Horgan and co-workers in 1957 (23) and Puig Muset in 1959 (24) reported that lipid peroxides are toxic to animals. The present studies have revealed that adult rat brain contains products of lipid peroxidation, the amount of which is only slightly affected by lack of vitamin E. A somewhat similar finding has been reported by Bieri (25). The relatively high level of these lipid breakdown products and the large quantity of PFA, - the major substrate for peroxidative reactions - in rat brain stimulated an investigation into the production and concentration of these end-products in the maturing rat brain.

II. LITERATURE REVIEW

Essential fatty acids have been implicated as structural units of great importance in animal tissues. The phospholipids of cell particulates are very rich in highly unsaturated fatty acids, particularly arachidonic acid. These unsaturated fatty acids are very susceptible to oxidation, and their in vivo integrity depends, therefore, on the presence of some stabilizing factor. In what follows, the literature concerning the occurrence, the nature, and the distribution of these unsaturated fatty acids; the nature of their oxidation and the role of vitamin E in maintaining their stability in animal tissues has been reviewed. In addition, an evaluation of the methods reported in the literature for the detection of PFA, peroxides and breakdown products has been undertaken.

A. Tissue Polyunsaturated Fatty Acids

1. Source

It has been shown that certain highly unsaturated fatty acids, namely, linoleic, linolenic and arachidonic, are essential dietary factors (26,27,28), while saturated, monounsaturated and very highly unsaturated fatty acids are not. The three EFA are not equally potent, however. For example, linoleic acid is effective in prompting growth, and in curing dermatitis and other fat-deficiency symptoms. Linolenic acid has some growth promoting activity, but is unable to prevent or cure the skin abnormalities.

Mead and co-workers (29,30), Reickehoff and co-workers (31), and others have demonstrated that linoleic acid is not synthesized in animals but that arachidonic acid can be synthesized in vivo from linoleic acid. Therefore, linoleic acid appears to be the primary essential factor, and must be supplied in the diet. Hansen et al. (32) reported that addition of linoleic acid as trilinolein to the diet corrected the skin lesions (characteristic of EFA deficiency) in infants who had been fed on a diet low in fat. Analyses of human blood lipids have shown that there are significant changes in the proportions of UFA, when the intake of linoleic acid is limited (33). Because two double bonds are present in the molecule, linoleic acid can exist in several different isomeric forms, and Privett et al. (34) have reported that the naturally occurring cis-isomer has the greatest potency.

Various vegetable oils are the richest source of EFA. For purposes of comparison, the compositions of representative fats of vegetable and animal origin are listed in Table A.

Table A
Fatty Acids as Percent of Total Acids (35)

Fat	Saturated	Oleic	Linoleic	Linolenic	Arachidonic
<u>Vegetable</u>					
Coconut oil	80	7	2	-	-
Corn oil	13	23-40	56	0.6	-
Cottonseed oil	26	27	47	-	-
Linseed oil	6-12	13-31	10-27	30-64	-
Margarine oil	23	62	5.8	0.1-0.9	0
Olive oil	8-16	53-86	4-20	-	-
Peanut oil	17	54	29	-	-
Soybean oil	15	25	55	0.3-10	-
<u>Animal</u>					
Butter	46-48	-	4	1.2	0.2
Lard	43	46	10	0.5	0.5
Shortening	25	62	5	0.2-0.6	0.5
Tallow (Beef)	53	42	4	0.5	0.5

The richest sources of linoleic acid are corn, cottonseed, and soybean oils. Of the animal fats, lard has a fair amount of linoleic acid.

Available experimental evidence seems to indicate that equally satisfactory growth in rats is obtained with a variety of vegetable oils and with butter. Thus Deuel et al. (36) observed that the extent of growth in rats during the first twelve weeks after weaning was the same, regardless of whether corn, cottonseed, peanut or soybean oil; butterfat or margarine was chosen as the source of dietary fat.

2. Distribution in animal body

Most of the studies described in the following sections were done on

brain and heart tissue of the rat. It would seem appropriate, therefore, to summarize here such information as is available regarding the PFA content of these organs.

Brain: The brain constitutes about 2% of the body weight in man, and the spinal cord and nerves account for an additional 0.4%. In the rat, the brain accounts for about 0.5% of the body weight. Most of the PFA of the brain are present as phosphatides. Welch (37) has reported that rat brain phosphatides have the following composition:

Lecithins 57% of total phosphatides

Cephalins 33.5% of total phosphatides

Beef brain phosphatides have a similar composition (37).

Klenk (38) reported the fatty acid composition of the ester phosphatides, which is summarized in Table B:

Table B

The Composition of the Fatty Acid Mixture Separated from the
Ester Phosphatides of Rat Brain

Type of acid	No. of Carbons	Fatty Acid Composition*		
		Lecithin	Phosphatidyl- ethanol-amine	Phosphatidyl serine
Saturated (total)	14 to 22 even-numbered	39.2	21.8	35.9
Unsaturated	16	1.2	2.3	-
	18	48.0	34.7	51.6
	20	7.3	15.1	7.1
	22	4.3	24.4	5.4
	24	-	1.7	-

* Expressed as percent of the total fatty acids isolated from each fraction.

The C₁₈ fraction consists almost entirely of linoleic, linolenic, and oleic acids. Beauvallet observed a relatively high proportion of arachidonic acid in the phospholipids of brain, although the other C₂₀ unsaturated acids listed below have also been found:

	Δ^{11}	Eicosenoic
	$\Delta^{11,14}$	Eicosdienoic
	$\Delta^{5,8,11}$	Eicostrienoic
	$\Delta^{5,8,11,14}$	Eicostetraenoic
and C ₂₂	$\Delta^{7,10,13}$	Docosatrienoid

The UFA present in the phospholipids of the white and gray matter have been shown to differ with respect to their degree of unsaturation(39). Thus the iodine value of the UFA isolated from the phospholipids of frontal whole matter was found to be 84, as compared with a figure of 129 for the phospholipid UFA prepared from frontal gray matter.

It has been pointed out in the literature that heart and liver have the same composition of fatty acids. Irving and Smith (40) found the total mixed fatty acids in pig liver to have the following composition:

Saturated:	(C ₁₂ ,C ₁₄ ,C ₁₆ ,C ₁₈ and C ₂₀)	34.8%
Unsaturated:	Palmitoleic	1.5%
	Oleic	28%
	Linoleic	5%
	C ₂₀	20%
	C ₂₂	7.5%

The distribution of lipids in rat brain has been studied by Biran and Bartley (9) and their findings are summarized in Table C.

Table C

Distribution of Lipids in Rat Brain and in Particulate Fraction Isolated from Brain Homogenates

Fraction	Total Lipid	Phospholipid	Neutral Fat
Homogenate	39.5	22.9	0.8
Mitochondria	51.7	25.8	3.6
Microsomes	34.2	22.2	1.1

*Values given are in mg. lipid per 100 mg. dry weight of tissue.

Mitochondria have the highest concentration of neutral fat while the phospholipid levels of the mitochondria and microsomes appear to be about the same. Total lipid of the brain homogenate and these particulate fractions were shown to have similar proportions of lecithin and cephalin. The distribution of fatty acids in whole brain homogenates, and in mitochondria and microsomes isolated therefrom, conforms to the same general pattern. Table D contains illustrative data compiled by Biran and Bartley (9).

Table D

n=carbon atom x=double bond	Fatty acid	Percent of total FA		
		Whole brain	Mitochondria	Microsomes
n - x				
n - o	Myristic } Palmitic } Stearic }	46.7	50.5	59.3
18-1	Oleic	30	31.9	24
18-2	Linoleic	1.4	-	-
20-1	-	3.7	1.6	1.4
20-4	Arachidonic	9.5	7.1	6.8
22-6	-	7.6	4.3	6.8
n - x>2		17.0	11.4	13.5

The proportion of arachidonic acid is about the same in the mitochondrial and the microsomal fractions. These findings are similar to those of Macfarlane et al. (41) for rat liver.

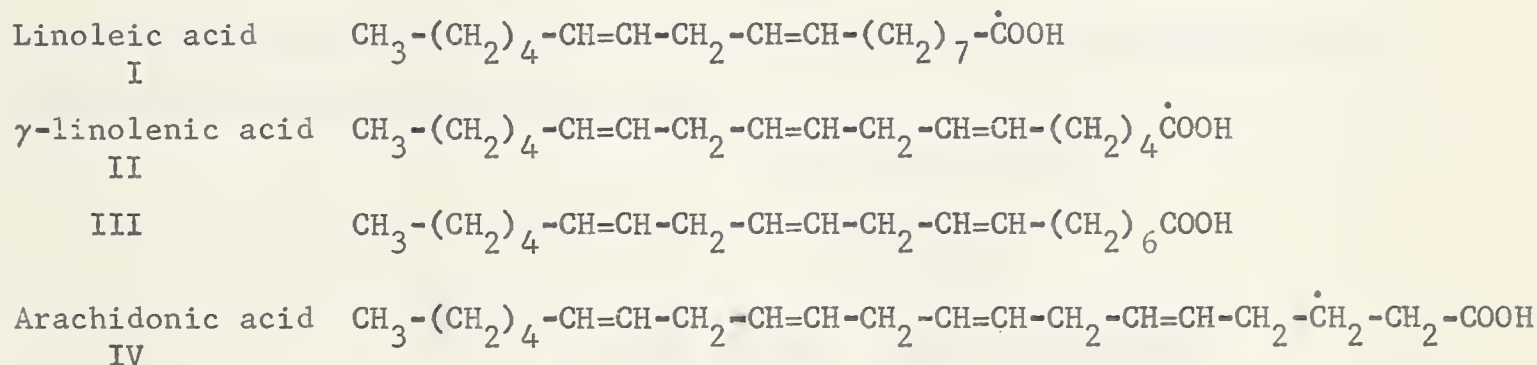
3. Biosynthesis

It has long been recognized that animals can synthesize saturated fatty acids from carbohydrates, through the two-carbon intermediate. Moreover, it was convincingly demonstrated by Schoenheimer and Rittenberg, that the mouse is able to bring about the desaturation of stearic into oleic acid. Stetten and Schoenheimer (42) demonstrated that palmitoleic acid is formed from palmitic acid, while Anker (43) showed that myristic acid can be desaturated in vivo to yield myristoleic acid.

Bernhard and Schoenheimer showed that the rat is unable to synthesize either the diethenoid acid, linoleic or the triethenoid acid, linolenic from carbohydrate. However, there is excellent evidence that linoleic acid can be transformed to arachidonic acid in the animal body. Mead et al. (29) injected rats previously fed carboxyl-labeled linoleic acid, with acetate-1- C^{14} . The arachidonic acid subsequently isolated from their organs was degraded stepwise, but no activity was found beyond the third carbon atom. Klenk has also reported similar findings.

This and other evidence indicates that although linoleic acid is not synthesized in the animal body, arachidonic acid can be synthesized from linoleic acid. Linolenic acid does not behave as a typical essential fatty acid in feeding experiments (Greenberg et al. (44) and Deuel et al. (45)).

Mead and Howton (46) have suggested that linoleic acid is transformed to arachidonic acid by the following sequence of reactions. Their conclusions are based on studies with C^{14} -labeled intermediates.



Transformation of linoleic to arachidonic acid

The acid designated by III has not yet been identified.

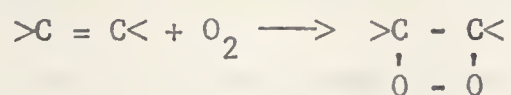
B. Oxidation of Unsaturated Fatty Acids

Polyunsaturated fatty acids are highly susceptible to oxidation because of their unsaturation. Three types of PFA oxidations have been reported in the literature: autoxidation, hematin catalyzed (or catalytic) oxidation, and lipoxidase catalyzed (enzymic) oxidation.

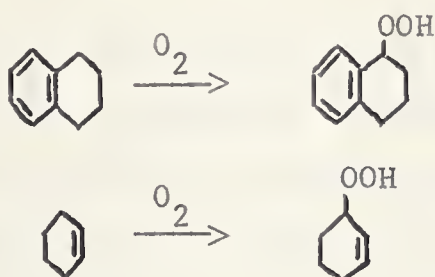
1. Autoxidation

It has long been known that unsaturation decreases during the autoxidation of unsaturated compounds. For many years this was interpreted as indicating that the primary reaction was an addition of O_2 to a double bond

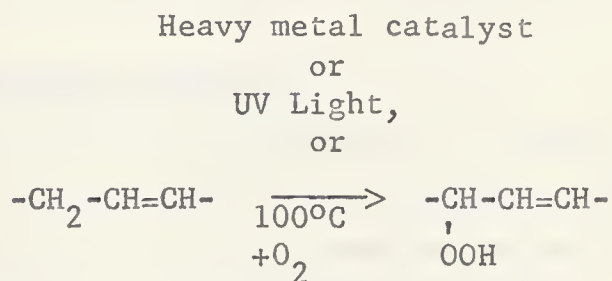
forming a cyclic peroxide, as proposed by Engler and Bach:



Such a cyclic peroxide has neither been isolated nor shown conclusively to occur. Furthermore, investigations of the autoxidation of numerous cyclic compounds with isolated double bonds yielded quite different results. In a great many cases the primary product was isolated in pure form and shown to be a hydroperoxide, with the peroxidic group in the α -position with respect to the double bond. Some of the hydroperoxides that have been isolated are:



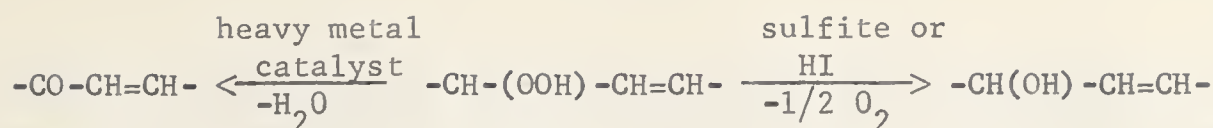
These findings have led to the formulation of the following scheme for the initial step in the oxidation of PFA:



Most of the preliminary work in this area has been reviewed by Gee (47) and Farmer et al. (48,49). The unsaturated hydroperoxides vary greatly in their stability, but generally decompose at elevated temperatures (above 100°), to yield a complicated mixture of decomposition and polymerization products.

Reduction with agents such as hydroiodic acid, sulfite, or aluminum amalgam in acetic acid, often gives high yields of the corresponding α - β unsaturated alcohol.

Heavy metals which catalyze the formation of these peroxides also catalyze their decomposition. The predominant reaction product is then generally the α - β -unsaturated ketone.



Ketones and alcohols are considered to be the main end-products of autoxidation. During decomposition of the hydroperoxides, especially at high temperatures, the double bonds, in large measure, disappear, due to epoxide or glycol formation, to chain splitting at the double bond with the production of carbonyl or carboxyl groups, or to polymerization.

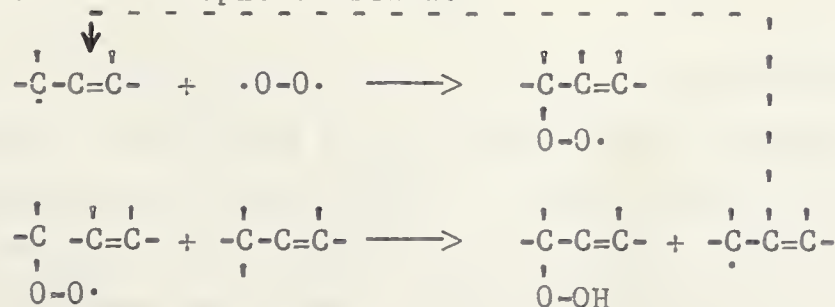
Since these reactions proceed at different rates at various temperatures and in the presence of traces of different catalysts, the difficulty of defining the individual steps of the autoxidation process is readily appreciated.

Holman and associates (50), demonstrated that the autoxidation of mono-ethanoid oleic acid at room temperature is extremely slow, and that in order to get the reaction to proceed at a reasonable rate it was necessary to employ heavy metal catalysts, u.v. light or higher incubation temperatures.

It has been shown (51) that at 37°C, in air, and in the absence of catalysts, the peroxidation of PFA required a long induction period, the length of which decreased with increasing degree of unsaturation of the fatty acid.

1. Mechanism of the autoxidation reaction

Farmer et al. (52) suggested that the autoxidation of unsaturated substances proceeds by addition of O₂ to an α-methylene group by means of a free radical mechanism. He postulated that the reaction sequence is triggered by the removal of a hydrogen atom from an α-methylenic position, followed by the reactions depicted below.

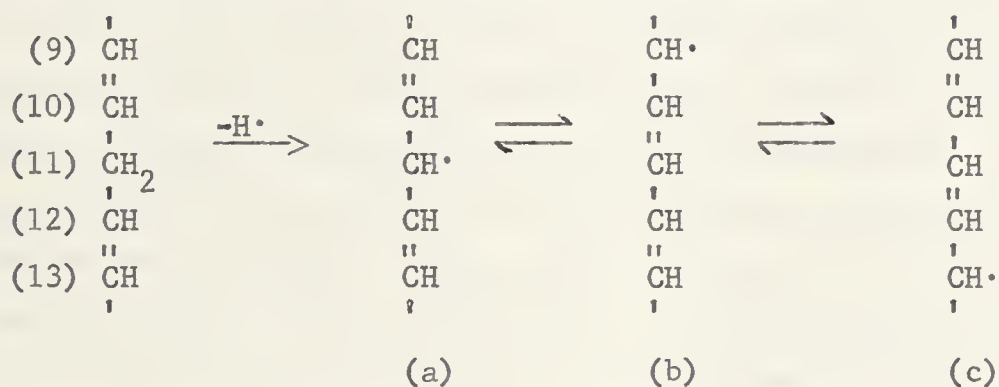


Because of resonance of the postulated intermediate, the free radicals that would be formed by the removal of H atoms from the C₈ and C₁₁ α-methylenic

groups of oleic acid, would contain four positions (C_8, C_9, C_{10} and C_{11}) at which oxygen could react to give a hydroperoxide. Although not much is known about the products arising from the autoxidation of oleic acid, the mechanism advanced by Farmer and associates is substantiated by the experimental evidence cited below.

In 1943, Farmer and Sutton (53) observed that the autoxidation of fish oil fatty acids containing unconjugated systems of double bonds was accompanied by an increase in the absorption at 230-240 $m\mu$. A similar observation had been recorded earlier by Ellis (54), who failed to relate it to the process of autoxidation. Farmer and co-workers (52) showed that in the early stages of autoxidation of methyl linoleate and methyl de-cosa-hexaenate an absorption band at 234 $m\mu$ appeared, the intensity of which increased in parallel with oxygen uptake and peroxide formation. They explained these observations on the basis of the mechanism of autoxidation, proposed by them earlier, which is given below.

Removal of a hydrogen from the methylene group at C_{11} in linoleic acid would yield a free radical that would be stabilized through resonance with the three contributing structures a, b and c:



The free radicals then react with O_2 to form hydroperoxides. Thus the hydroperoxides formed from b and c would show conjugated double bond systems. A conjugated double bond system is known to give absorption at 234 $m\mu$. Farmer and associates (52) reported that in the oxidation of ethyl linoleate, the monohydroperoxide formed, contained approximately 70% of conjugated diene isomers. This supports their theory of the mechanism of autoxidation.

The removal of a hydrogen atom from the central methylene group of a 1,4-diolefin should be considerably easier than the removal of a hydrogen

atom from an ordinary α -methylenic group by virtue of the greater resonance energy of the former radical. Orr calculated their resonance energies to be 30 and 19 kg.-cal. per gram mole, respectively. This appears to be the main reason for the higher rate of autoxidation of the 1, 4 diolefins. The conjugation of the double bonds can only take place when they are separated by a single methylene group. No conjugation is observed in the autoxidation of squalene or natural rubber, in both of which the double bonds are separated by two carbon atoms.

Lundberg and Chipault (55) observed that some secondary reactions occur even in the earliest stages of fatty acid peroxidation at various temperatures (40, 60, 80 and 100°C). They found that a small fraction of the absorbed oxygen was not present as peroxide and that the magnitude of this fraction increased with increasing temperature. They also detected the presence of secondary products, with absorption maxima at 277.5 m μ .

Bolland and Gee (56,57) have carried out a careful kinetic and thermochemical analysis of the early stages of the autoxidation of ethyl linoleate under different conditions and all the data they obtained was compatible with the free radical mechanism proposed by Farmer. The oxygen absorbed was proportional to the peroxides formed. Further evidence for the free radical mechanism is that, as in other autoxidations, substances that are known to decompose and yield free radicals, catalyze the reaction. Bergstrom (58) has investigated the reaction of the 1,4-diolefin system with N-bromosuccinimide and selenium dioxide. When methyl linoleate in carbon tetrachloride solution was reacted with this reagent, a strong absorption maximum appeared at 232 m μ , indicating the generation of a conjugated double bond system. On prolonged boiling the bromine was split off with the formation of a triene. Since bromination with reagents of this type shows all the characteristics of a free radical reaction, these results lend further support to the previously described autoxidation mechanism.

Bergstrom (59) was able to isolate the C₉ and C₁₃ hydroxystearates present in hydrogenated autoxidized linoleate, although no C₁₁-hydroxystearate was found. Previously, Farmer had shown 70% conjugated diene isomers to be present in autoxidized ethyl linoleate. Khan, Lundberg and Holman (60) have recently studied the infrared spectra of linoleate hydroperoxide preparations

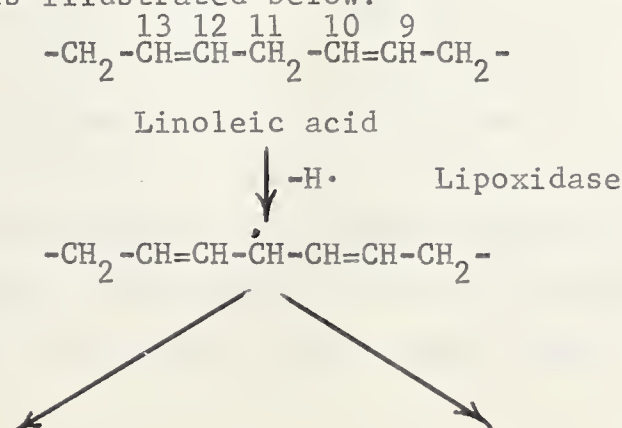
of high purity and have found that the product is at least 90% conjugated and that it consists largely of cis, trans isomers. This work has been confirmed by Sutton and Sephton (61), who have also shown that the geometrical forms obtained during autoxidation of methyl linoleate at ordinary temperatures are largely conjugated cis-trans forms. Some trans-trans forms arise partly, at least, by thermal rearrangement of the already-formed cis-trans peroxides.

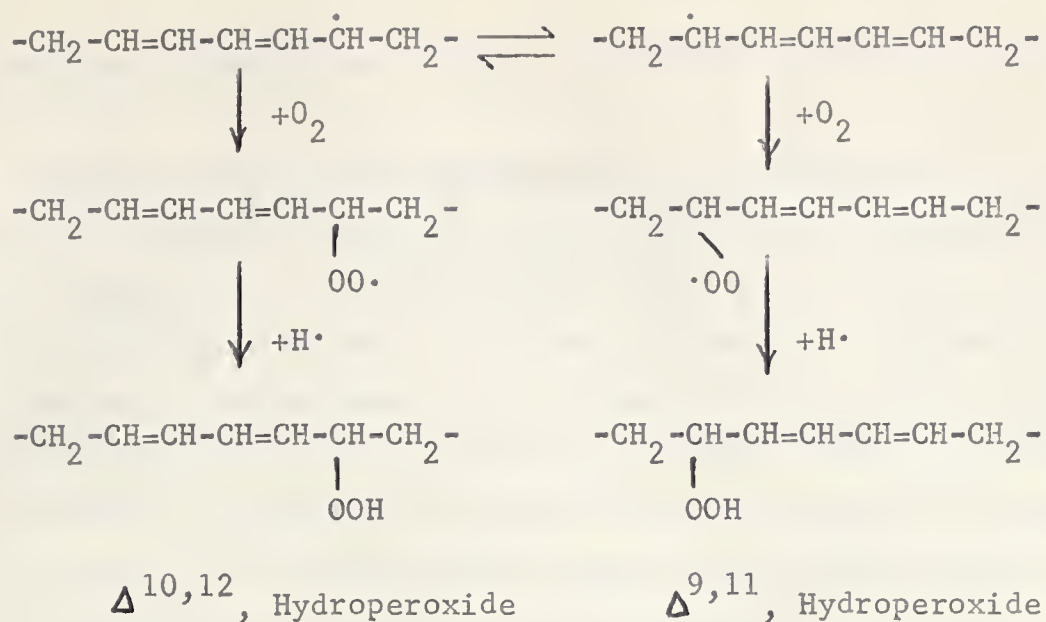
Thus, the primary steps in the autoxidation of the UFA appears to be quite clear, due largely to the work of Farmer, Bergstrom, Gee and Koch and associates. However, much less is known about the subsequent reactions in which the peroxides decompose to yield a complicated mixture of more stable non-peroxidic compounds, including carbonyls, epoxides, alcohols and polymerized products. The latest developments in the study of the breakdown products of peroxides are discussed in the following section.

2. Hematin and lipoxidase catalyzed oxidation of PFA

Lipoxidase is an enzyme which catalyzes the oxidation of PFA. It is widely distributed in the plant kingdom, having been found in soybeans, white lupine, alfalfa, potatoes; and in seeds of a variety of legumes. However, attempts to demonstrate the presence of the enzyme in animal tissues have failed. The purified lipoxidase was reported by Holman and associates (62) to have a molecular weight of 102,000 and a pI at a pH of approximately 5.4. The same investigators (63) have also estimated that the crystalline enzyme contains 938 amino acid residues per molecule.

The mechanism of the lipoxidase catalyzed oxidation of PFA has been discussed by Bergstrom and Holman. The mechanism that they have proposed is very similar to the one which has been advanced to describe the events of autoxidation, and is illustrated below:





The oxidative rancidity produced by deterioration of PFA during storage of meats has been an active field of research for some time. The presence of unsaturated fatty acid oxidases in animal tissues has been suggested occasionally. Banks (64) demonstrated the presence of a heat-labile system in herring muscle, which stimulated the development of rancidity in herring oil. However, the active substance was subsequently shown to be a hematin protein and its activity shown to be quite different from that of lipoxidase. The herring muscle preparation, unlike lipoxidase was optimally active at low pH (65). The behavior of the system described by these workers was similar to that of the system in which the in vitro oxidation of linoleate is stimulated by hemin.

A rancidity-promoting 'enzymic' activity in pig-muscle (Lea (66)) was later proved by Watts and Peng (67) to be due to the Hb and Mb of the muscle. Hove (68) has reported the presence of a fatty acid and oxidase in rat liver and gastric mucosa, and Reiser (69) has suggested that UFA oxidation is enzyme catalyzed. The latter worker's conclusions were based largely on his observations that Hb-free extracts of unsmoked bacon adipose tissue contained a heat labile component which catalyzed the oxidation of lard.

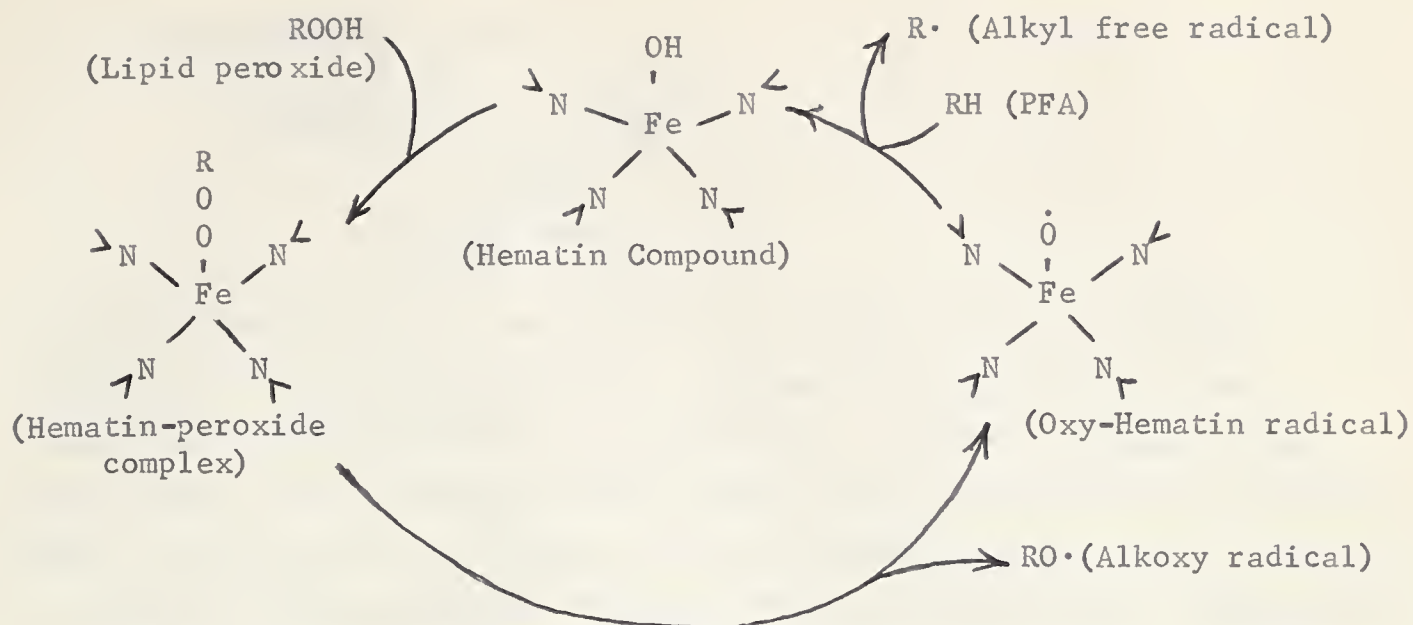
Whether or not these enzymes (if indeed, they occur at all) bear any resemblance to lipoxidase is not clear. Nor has any convincing evidence been advanced to indicate that they play a significant role in animal

metabolism. None of the 'enzymes' described has been either characterized or purified.

Tappel in 1952 (70) suggested that the heat lability of linoleate oxidation catalysts in muscle extract might be due to an effective removal of heme compounds by protein coagulation, rather than to a denaturation of enzymes capable of direct oxidation of linoleate. Tappel (71) reported the absence of lipoxidase in the tissues of rat, cattle, chicken, turkey, and various fish. He also reported (72) that hemin, Hb, and cytochrome-C are powerful catalysts for the oxidation of colloidal linoleate. The rate of oxidation of the PFA of pork adipose tissue, more rapid than that of a simple autoxidation process, can be explained on the basis of its content of hematin compounds. Linoleate oxidation catalyzed by hematin compounds has a relatively low activation energy of 3.3 Kcal. per mole. The catalytic activity of iron containing proteins decreases in the order (73): cytochrome > hemin > Hb > catalase.

Considering that PFA react directly with molecular O_2 and that cytochromes and other hematin compounds are active catalysts of lipid peroxidation in animal tissues, one might expect that the sub-cellular particles containing these PFA would be labile to oxidative deterioration. Ottolenghi et al. (74) have, in fact, shown that U.V. light and added ascorbic acid initiate lipid peroxidation in isolated rat liver mitochondria, thus causing inactivation of certain of the mitochondrial enzymes.

Tappel and Zalkin (6) suggested that lipid peroxidation in suspensions of isolated liver mitochondria is mainly hematin catalyzed, since lipid peroxidation in this particulate fraction is inhibited by cyanide ions and by methylene blue--both selective inhibitors of hematin. They suggested that hematin-catalyzed peroxidation is the basic mechanism underlying both the in vivo pathological reactions found in vitamin E deficient animals, and the development of rancidity in food. Maier and Tappel (75) have proposed a mechanism for hematin catalyzed lipid peroxidation. They suggest that the process is initiated by a reaction between the hematin compound and the hydroperoxide of the fatty acid--a reaction in which free radicals are formed as depicted below.

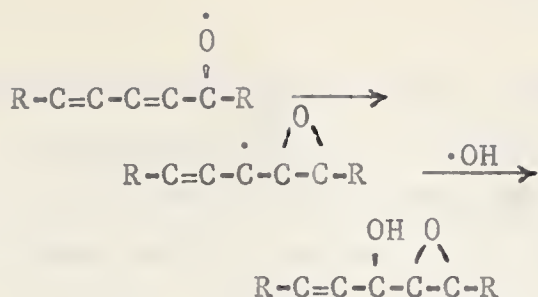


The free radicals formed in the above scheme can react in a number of ways, depending upon the environmental conditions during the reaction. The main pathways under conditions commonly occurring in the presence of excess PFA appear to involve a free radical chain.



The first cycle of the chain reaction is thus completed. The oxyhematin radical may react with lipid to initiate another reaction chain of the same kind. Maier and Tappel were able to trap alkoxy free radicals by reducing them with hydroquinone, to the corresponding alcohol, and thus to identify them.

The free radical produced by the direct decomposition of hematin lipid peroxide complex may also be involved in a concurrent scission of the -C-C- or -C=C- bonds in the lipid giving other products, mainly carbonyl compounds. Other secondary products were detected which contained groups like oxirane and hydroxyl with loss of conjugated double bonds. Such compounds may be formed by the intramolecular attack of an alkoxy radical on an adjacent double bond, followed by radical combination between the alkyl radical produced and a hydroxyl radical:



The suggestion that lipid peroxidation in animal tissues is hematin catalyzed, is a very attractive one. Holman (76) reported that pure lipoxidase is not inhibited by pyrophosphate, fluoride, cyanide, azide, mercury ions, etc. One would expect then, that if lipoxidase or a similar enzyme were operative in tissues, lipid peroxidation would not be inhibited by cyanide or methylene-blue. However, Tappel and co-workers observed that these compounds did, in fact, inhibit lipid peroxidation.

Tappel and Zalkin (6) observed that both O_2 uptake and PFA oxidation by mitochondrial suspensions in which the hematin had been specifically immobilized, were sharply inhibited. They felt that the slight residual PFA oxidation was due to autoxidation, and interpreted their results as indicating that fatty acid peroxidation in tissues is primarily a hematin catalyzed reaction. Furthermore, using linoleate as the model unsaturated fatty acid, the activation energies for hematin catalyzed oxidation and autoxidation were 3.3 and 15 Kcal./mole respectively. The lipoxidase catalyzed oxidation of linoleate was found to have an activation energy of 4.3 Kcal./mole (Tappel (73)).

C. Role of Vitamin E in the Maintenance of Tissue Lipid Stability

Vitamin E appears to be essential for the maintenance and normal function of several animal tissues. The 'clinical symptoms' associated with a deficiency of this vitamin differ from one species of experimental animal to another. Some of the more thoroughly documented ones are as follows:

(1) Dam in 1944 (77) described encephalomalacia, a condition which involves impairment of essential tissue function, and in which large areas of brain tissue fail to develop or are destroyed in the encephalic syndrome. Encephalomalacia only occurs in chickens of a certain age group, and is related to the stage of development of the brain (5). Exudative diathesis, according to these workers, is characterized by edema, which appears first in adipose tissue around the crop, then under the skin and in the muscles.

The exudate has the same protein content as plasma. When it first appears it has a green color, which subsequently disappears, after which the exudate coagulates and is sometimes reabsorbed. Recurrence of exudates is common; when severe, exudative diathesis damages breast and leg muscles and leads to death. The basic abnormality is an increased capillary permeability. The appearance of the exudate is accompanied by the development of peroxides in the subcutaneous fat.

(2) Effect on reproduction

Testicular degeneration has been noted following the development of a vitamin E deficiency in rat and many other species, with the exception of the mouse (78,79). Evans and Burr, as early as 1927, described fetal resorption in vitamin E deficiency, and noted that it could be prevented by the administration of vitamin E early in the gestation period. Blandau *et al.* (80) have expressed the opinion that this deficiency symptom reflects a failure of uterine rather than ovarian function.

(3) Muscular dystrophy

The histological deviations from normal muscle structure in vitamin E deficiency reflect a vitally important function of tocopherol, but the mechanism of its action in this regard is not completely understood. Mackenzies and McCollum (81,82) were the first to establish the fact that vitamin E deficiency led to the development of muscular dystrophy in rabbits and guinea pigs. The gross and microscopic changes which accompany the development of dystrophy in young adult and in mature rabbits have been recorded. The paresis is characterized by an inability to walk or stand, flabbiness and weakness of the muscles, dragging of the hind legs, pronounced muscle atrophy, deformation of the feet, etc. Death is usually attributed to the paralysis of the respiratory muscles. The symptoms of 'stiff lamb disease', and of white muscle disease in lambs and calves (83,84) have been reported to bear a close resemblance to those of muscular dystrophy.

(4) Pigmentation of adipose tissue fat in the rat

The group of Granados, Mason and Dam, in a series of papers, reported the development of a brownish pigment in animals fed a vitamin E deficient diet containing a high level of polyunsaturated fatty acids. Harris *et al.* (85) has suggested that the most likely explanation for this phenomenon is

that the unsaturated fats become deposited in adipose tissue cells which do not have enough tocopherol to stabilize them (i.e. to block peroxide development). The peroxides then polymerize or combine with cell proteins, or both, to produce acid-insoluble pigments. This suggestion finds support in the work of Tappel (86).

Evidence Implicating Vitamin E as an Essential Factor in the Maintenance of Fat Stability in vivo

One of the chief properties of PFA is the readiness with which they undergo autoxidation. Several investigators have examined the possible role of such oxidations in the normal metabolism of PFA. Dubouloz (87) and his associates reported that peroxides of the fatty acid esters formed after the ingestion of ethyl oleate are gradually eliminated from the gastrointestinal tract, and that no determinable quantities are deposited in the tissues. Although small amounts of such compounds (peroxides) apparently do occur in adipose tissue, resulting in its well-known brown appearance, their digestive origin has not been proven. The action of x-ray irradiation has been shown to result in the formation of lipid peroxides in the skin of the rat (88).

Dam and Granados (1) found that there was an increased peroxide content in the fat of several organs of vitamin E deficient chicks and rats. It had earlier been shown by Burr et al. (89,90,91) that fat from rats and hogs on vitamin E deficient diets autoxidized more rapidly in vitro than did fat from normal animals. Moreover, the autoxidation process did not exhibit an induction period with fat from the vitamin E deficient animals, as it did with fat from normal animals.

Lipid peroxides can cause much structural and metabolic damage. Horgan et al. (23) have demonstrated the toxicity of the injected lipid peroxides. Studies comparing the toxicity, in mice, of x-ray irradiation and of injected peroxides, have led to the hypothesis that radiation toxicity in mammals is due to the initiation of autoxidation of PFA producing lethal doses of peroxides in sites not reached by vitamin E. Andrew et al. (92) found that the concentration of a toxic principle present in air-oxidized soybean oil, corresponded closely to the peroxide concentration of the oil. Absorption studies indicated that, although the reduced products of the peroxides were

absorbed, the peroxides themselves were destroyed in the intestine. The toxic action of the lipid peroxides seemed to take place at the level of the intestinal enzymes. Ottolenghi et al. (74) had previously observed the toxic effect of peroxides induced by radiation. Poor reproductive performance in rats fed irradiated, semi-synthetic diets might be attributable to vitamin E destruction. The effects of several antioxidants on the survival time of mice exposed to multiple, sublethal doses of total body x-irradiation, have been investigated. Ershoff and Steers (93) observed that propyl gallate and 2,5 di-tert butyl-hydroquinone, at levels of 0.25 or 0.5% in the diet, increased the mean survival time of irradiated mice as compared with that of animals maintained on unsupplemented rations. Mixed tocopherols and santoquin at the former level, and DPPD at both levels had little, if any protective effect.

Tappel (86) has reported the in vitro production of yellow-brown co-polymers having properties similar to those of the pigments which have been found in the adipose tissues of vitamin E deficient rats. He obtained these co-polymers by in vitro oxidation of emulsions of linoleic acid or cod liver oil in the presence of proteins. Hematin compounds, particularly hemoglobin, were shown to catalyze the co-polymer formation. He suggested that the in vivo changes in catalytic and structural proteins, due to vitamin E deficiency, may result directly from the reaction of unsaturated fat oxidation products with proteins, and that vitamin E may prevent these changes by functioning primarily as an unsaturated fat antioxidant.

Maier and Tappel (94) demonstrated inhibition of hematin catalyzed oxidation of cod liver oil by malachite green, MB, rosaniline, etc. The ability of these redox dyes to replace part of the vitamin E in the diet of rats is probably a function of their antioxygenic properties. Many such reports are available but it is not possible to describe all of them here. However, it is interesting to note that Schwarz (95) evaluated thirteen antioxidants at various dose levels for effectiveness in protecting rats against necrotic liver degeneration. Di-tert-amyl-hydroquinone, santoquin and DPPD were effective, but ascorbic acid and MB afforded no more than 30 to 40% protection.

Uri (96) discussed the inhibition of fat oxidation and concluded that although α -tocopherol is a reasonably good antioxidant, it is inferior to butylated hydroxy anisole, propyl gallate or the naturally occurring or synthetic flavanols. However, α -tocopherol has the advantage of passing from the food into animal body fat. Other forms of tocopherol, such as δ -tocopherol, have much higher antioxidant activity than does α -tocopherol. Collier and McRae (97) reported that hemolysed human erythrocytes catalyzed the oxidation of linoleate at pH 7. This oxidation was shown to be due to Hb and not to the presence of a lipoxidase. The catalytic action of erythrocyte hemolyzates and of pure Hb, was inhibited by α -tocopherol and α -naphthol. It is possible that vitamin E plays a role in maintaining the integrity of erythrocytes by inhibiting the Hb-catalyzed oxidation of UFA of the cell membrane.

Hamilton et al. (98) reported that hamsters on a vitamin E deficient diet grew slowly and that deficiency symptoms became acute in 4-16 weeks. The animals made a spectacular recovery on the injection of 5 mg. of α -tocopherol. The deficient animals were severely hemorrhagic. Day and Dinning (99) observed a similar improvement in young Rhesus monkeys following α -tocopherol treatment. Exudative diathesis in chicks was prevented by α -tocopherol (Scott et al. (100)). No evidence of liver necrosis was noted in vitamin E deficient birds.

Christensen, Gortner and Dam (101) have described the in vitro hemolysis of erythrocytes from vitamin E deficient rats and chicks. Hemolysis was achieved merely by suspending the erythrocytes in isotonic NaCl solution at 37°C. Dialuric acid was not required for hemolysis of the vitamin E deficient erythrocytes. Dinning (102) reported that rats, after 5 months on a vitamin E deficient diet, showed no gross deficiency signs and no marked growth inhibition. On the other hand, he reported a greater turnover rate of nucleic acids, based on the increased C¹⁴-formate incorporation into these macromolecules.

Rosenkrantz (103) reported an increased respiration rate in the adrenal cortex of rabbits maintained on a vitamin E deficient diet for 14 days. The respiration rates of skeletal muscle and liver tissue from vitamin E deficient animals were elevated above the normal, while those of heart and kidney

tissues from vitamin E deficient and normal animals were essentially the same. Mertz and Schwarz (104) reported a decline in the O_2 consumption by liver slices from vitamin E deficient diet, which could be reversed by vitamin E addition to the slices in vitro.

Bernheim and Ottolenghi (105) found that no fatty acid peroxides were formed in rabbit bone marrow homogenates incubated in vitro, and that marrow extracts inhibited peroxide formation in rat liver homogenates. The extract also prevented the inhibition of liver succinoxidase activity by added peroxides. Marrow from irradiated animals, however, did produce peroxides when incubated in vitro and was less effective in blocking the inhibition of succinoxidase by added peroxides. Vitamin E inhibited peroxide formation in liver homogenates, the degree of inhibition being proportional to the concentration of the vitamin over the range 10-100 $\mu\text{g/ml}$, but did not protect succinoxidase from inhibition by added peroxides. This suggests that it is the peroxides which inactivate succinoxidase, and that vitamin E in normal animals protects this activity by preventing the formation of peroxides.

Sinclair (106) commented on the relationship between vitamin E and PFA. "The roughly parallel distribution of linoleic acid and of tocopherols in vegetable oils is fortunate since it tends to insure sufficient tocopherols to prevent any side effects from oxidation of linoleic acid, if unprocessed vegetable seed oils are included in the diet." Tappel and Zalkin (6), and Ottolenghi (74) reported that the livers of vitamin E deficient rabbits are damaged by lipid peroxidation, and that the lipids in mitochondria isolated therefrom are more susceptible to oxidation than are the PFA in mitochondrial suspensions prepared from normal rabbit livers. The presence of vitamin E or selenite in the diet was found to inhibit the peroxidation reaction (107). It is suggested that dietary selenite functions by forming lipid antioxidants in vivo.

Ascorbic acid synthesis in liver homogenates has been shown to be reduced by the presence of lipid peroxides. (108)

The experimental evidence cited in the preceding pages makes it tempting to speculate that one of the key functions of vitamin E is to stabilize unsaturated lipids against oxidative deterioration, thus maintaining structural

and functional integrity at the subcellular level.

Some workers have suggested that tocopherol functions as a co-factor in the cytochrome-C reductase system in the respiratory chain (Nason et al. (109,110). However, supporting experimental evidence for this claim is meagre. Attempts to restore the activity of iso-octane-extracted enzyme preparations have shown that the reactivation is not specifically dependent on α -tocopherol. Marinetti et al. (111) and Draper and Csallany (112) found that a great number of substances, chiefly of lipid nature, were active; for example, vitamin K, ubiquinone, phytol, neutral fat, Tween 80, etc. Pollard and Bieri (113) have further shown that physical manipulation such as freeze-drying, lyophilization, and centrifuging restore the activity in iso-octane-extracted enzymatic preparations.

Donaldson et al. (114) have described experiments with aged cytochrome-C-reductase preparations. They claimed that α -tocopherol had a specific reactivating effect. Pollard and Bieri (115), however, demonstrated that not only tocopherol, but certain antioxidants and phenolic compounds as well, were able to reactivate aged cytochrome-C-reductase preparations, and they postulated that the activity-depressing effect of aging was caused by the formation of lipid peroxides.

D. Evaluation of the Methods

During the early search for methods to estimate lipid stability, attempts were made to correlate changes in free fatty acid content, iodine value and the content of oxidized fat, to determine if these might serve as indices of autoxidation. There have been considerable improvements in recent years in the methods applicable to pure fats. For example, hydroperoxides can be estimated with reasonable accuracy by Lea's improved iodometric method and by the Ferric-thiocyanate method. Spectrophotometric estimation of hydroperoxides has been widely used and a polarographic method has recently been described. Spectrophotometric methods have been applied to studies of the breakdown products of peroxides.

However, most of these methods are not applicable to studies with animal tissues. In such investigations, autoxidation of the unsaturated fat has been most frequently followed by the measurement of oxygen uptake and the

results related to the amount of peroxides formed during that time, as measured either by the iodometric method or by estimating the carbonyl compounds produced by the Kreis method. (Carbonyl compounds yield a colored complex with phloroglucinol.) The measurement of O_2 uptake does not provide a quantitative estimation of the rate of lipid peroxidation in tissues, because of the utilization of O_2 due to tissue respiration. Wilke et al. (22) used nembutal (pentobarbital sodium) to completely inhibit respiration, and considered the residual O_2 uptake to be a measure of lipid peroxidation. However, the method is cumbersome and applicable only to in vitro studies.

Lea's iodometric method is not adequate because of its low sensitivity, and the ferric-thiocyanate method, although quite sensitive, is applicable only to purified extracted lipids.

The thiobarbituric acid test, originally used by Kohn and Liversedge (116), is now being widely used. It provides a very sensitive method for the estimation of one of the products of lipid peroxidation, - malonaldehyde. Thus, the estimation of MA produced gives a measure of the peroxide content of, and the extent of peroxidation in, a tissue. Because of its high sensitivity, the TBA method was accepted as the method of choice in the investigations described here.

Some of the 2-deoxy sugars, sialic acid, and certain other compounds yield malonaldehyde on periodic acid oxidation (Waravdekar and Saslaw (117)). However, these compounds do not interfere with the estimation of MA under the experimental conditions employed in these investigations. Recently Cawlishaw (118) has reported that haem gives a positive TBA test. This test therefore, is not applicable to RBC. Futterman and Saslaw (119) have used TBA to estimate vitamin A aldehyde. However, the reaction is carried out in presence of thiourea and in complete darkness--conditions quite different than those used in these studies.

The alkali-isomerization method (120) has been used extensively in estimating PFA. It is based on the absorption of U.V. light by the conjugated double bond system induced by alkaline isomerization of the PFA. Although the method is quantitative for cis isomers, trans isomers if present interfere. This fact imposes a severe limitation on its usefulness.

In 1959 MacGee (121) described an enzymatic method for the quantitative estimation of total PFA, which permits the precise estimation of as little as 5 γ of these compounds. This method was applied to tissue systems in the present work.

III. METHODS

1. Animals and Diets

Rats of the Wistar strain were used throughout this study and were allowed water and food ad libitum.

Although in some very early studies, Torula yeast was added to the deficient diet (30% w/w) to accelerate the vitamin deficiency symptoms, this addition did not markedly affect the biochemical findings (Pritchard and Nykiforuk, personal communication). All subsequent experimentation involved the use of a tocopherol deficient diet (Nutritional Biochemicals Corp., Cleveland, Ohio) of the following composition:

"Vitamin free"	Casein	20%	Brewers Yeast	10%
	Sucrose	56%	U.S.P.	
	Lard	10%	Salt mixture	4%

In the control animals this diet was supplemented with 10% w/w Rovamix E (Hofmann; LaRoche, Montreal, Quebec). In comparative studies, littermates were always used.

In corn oil experiments (Section B, p. 84) the littermates were apportioned as shown below:

- (1) Control diet
- (2) Vitamin E deficient diet
- (3) Control diet 4% w/w corn oil
- (4) Vitamin E deficient diet 4% w/w corn oil

In most of the studies reported in Sections A and C, the rats were maintained on a commercial Fox Chow diet supplemented with 10% Rovamix E. In studies with immature rats, the young were left with their mothers until being sacrificed at from 1-26 days postpartum.

2. Tissue Preparation

Animals were weighed, and killed by decapitation. The tissues were quickly excised, weighed and placed in ice-cold 0.05 M Tris^{*} buffer, pH 7.4, unless otherwise specified. The tissues were homogenized in glass homogenizers

* Trishydroxy methyl amino methane ($C_4H_{11}NO_3$).

and the homogenates adjusted to the desired concentrations. In most cases, brain and heart homogenates were made up to contain 50 and 25 mg. wet weight of tissue per ml., respectively. In the few instances in which other media were used for tissue preparation, their compositions are given as footnotes of the appropriate tables. The 0.05 M Tris buffer, pH 7.4, was prepared by dissolving 1.5125 gm. of Tris in 200 mls. of water, adjusting the pH to 7.4 with 1N HCl, and then making up the volume to 250 mls. with demineralized water. Fresh Tris buffer was made before each experiment.

Most of the studies were performed with brain and heart tissues. The term brain, as used here, refers to cerebral hemispheres, stripped of as much white matter as possible. All the other parts of the brain were dissected away.

3. Incubation

The tissue homogenates were placed in open, 25 or 50 ml. Erlenmeyer flasks and incubated at 37°C, in a Dubnoff metabolic shaker. Care was taken to use flasks of the same capacity for tissue homogenates and to use about the same volume of the tissue homogenate in each flask.

In general, the incubation time chosen throughout this study was either 180 or 120 minutes. In the experimental series in which the effect of incubation time on the production of TBA-positive material and PFA level was studied, incubation times of 1, 1-1/2, 2, 3, 4, 24, 48 and 72 hours were chosen.

4. Separation of Tissue Fractions

The separation of the subcellular fractions was effected by the Spinco Model L centrifuge, with a rotor head number 40. The speeds employed for different fractions are listed below:

<u>Fraction</u>	<u>Centrifugal force</u>	<u>RPM</u>	<u>Time</u>
a. Nuclear fraction	600 x g	3,000	15 min.
b. Mitochondria	8500 x g	11,300	15 "
c. Microsomes	100,000 x g	39,000	60 "
d. Supernatant fraction			

The supernatant fraction is the fluid portion left over after the removal of the microsomes. In certain experiments the microsomes were not sedimented and in these cases the fraction remaining after the removal of

the mitochondria was called the microsomal-supernatant fraction.

After each fractionation, the sedimented material was homogenized in 0.05 M Tris, pH 7.4, and the volume made up to the original volume of the whole tissue homogenate. This gave suspensions in which the concentrations of the individual particulates were approximately the same as they were in the original homogenate.

5. Lipid Extraction

Lipids from the tissue homogenates were extracted by the following procedure. Tissues were homogenized in 0.05 M Tris, to give suspensions containing 100 mg. wet weight of tissue per ml. Three mls. of homogenate and 6 mls. of ice-cold 20% trichloroacetic acid (TCA) were added to a centrifuge tube, kept in an ice bath. After mixing thoroughly with a glass rod for 5 minutes, the contents were centrifuged for 3 minutes in a clinical centrifuge. The supernatant, on which the TBA test was subsequently performed, was collected in a flask. This supernatant would be expected to contain water-soluble malonaldehyde, which is an end-product of the lipid peroxidation.

(a) The precipitated material in the centrifuge tube was then extracted with 5 mls. of 95% redistilled ethanol at 0°C. The ethanol extracts much of the lipids from the TCA-precipitated lipids and proteins mixture. After centrifugation, the ethanol extract was decanted into flask A.

(b) The precipitated material from (a) was treated 2 times with 5 ml. portions of 95% ethanol, heating each time for about 3 minutes in a water bath at 80°C. The ethanol extract was each time poured into flask A.

(c) Finally, the precipitate from (b) was mixed thoroughly with 5 mls. of petroleum ether and centrifuged. The ether extract was also poured into flask A.

The contents of flask A (the lipid extract) were reduced to dryness on a Rinco Rotary Evaporator, under reduced pressure. The residue was taken up in a volume of pure absolute methanol equal to that of the original tissue homogenate, usually 3 mls.

The extract thus obtained was tested for TBA-positive material, PFA content, and diene concentration.

6. Peroxidation Products

(a) Thiobarbituric acid test

Reagents:

Trichloroacetic acid: TCA, 35% w/v in demineralized water and 70% w/v in water.

Thiobarbituric acid: * TBA, 2 gms. of the 2-thiobarbituric acid were suspended in 193 mls. of demineralized water and 6.6 mls. of 2N NaOH added to it. This suspension was heated and stirred to give a clear solution. It was cooled and acidified with 0.7 ml. of 4N HCl. The yellow-colored solution thus obtained was decolorized with norite and filtered. This process was repeated until the reagent solution was colorless. This solution was stable for 1-2 days, after which the yellow color reappeared. Fresh TBA reagent was prepared for each experiment.

Method:

(i) The tissue homogenate contained in an Erlenmeyer flask was incubated at 37°C in a Dubnoff metabolic shaker, after two 2-ml. samples had been removed to serve as zero-time controls. Two 2-ml. aliquots were removed at various time intervals during incubation.

(ii) One ml. of 35% TCA was added to each 2-ml. sample contained in a 12 ml. centrifuge tube and the components mixed by swirling.

(iii) To this mixture were added 2 mls. of the TBA reagent, and the components were again mixed thoroughly by swirling. The samples were then placed in a boiling water bath for 20 minutes, the tubes being shaken occasionally during this period.

(iv) After 20 minutes the centrifuge tubes were taken out and 2 mls. of 70% TCA were added to each. After mixing the contents of the tubes, they were allowed to stand, with occasional shaking, for 25 minutes to permit color development.

(v) Three mls. of chloroform were then added to each tube, which was stoppered and shaken, and centrifuged for 5 minutes at 3,000 rpm in a clinical centrifuge.

(vi) A 4-5 ml. portion of the clear, aqueous supernatant solution was removed and its optical density at 535 mμ measured in a Klett-Somerson Photometer.

* $\text{NHCSNHCOCH}_2\text{CO}$ M.W. 144.16
[]

Results were usually expressed as absorbance per 100 mg. of tissue or per mg. of PFA in the tissue, or per whole organ. Absorbance was obtained by multiplying the Klett units by 0.002. The factor 0.002 is obtained from the expression: $\frac{1000 \times D}{2} = R$ where R = scale reading on the Klett-Somerson photometer, D = optical density. Occasionally, the results were also presented as TBA units, which are the same as Klett units, representing a peroxidation breakdown product, i.e. the TBA-positive material. Therefore

$$\text{Absorbance} = \frac{\text{Klett Units}}{\text{or TBA units}} \times 0.002 \propto \frac{\text{Peroxidation breakdown product}}{\text{or TBA - positive material}}$$

The levels of TBA-positive material, estimated on freshly precipitated, unincubated tissue homogenates, are considered to represent innate tissue peroxidation products or in vivo peroxidation products and are denoted in the tables by the zero time values. The homogenized tissues were usually incubated in air for 3 hours at 37°C. The amount of TBA-positive material in these homogenates after incubation provided an estimate of the susceptibility of the tissue lipids to peroxidation.

The TBA test was calibrated with pure malonaldehyde (Figure 1), and the MA-TBA pigment and the pigment produced by the reaction of TBA with the tissue TBA-positive material were both examined by the paper chromatographic method of Kitabchi and his associates (16) (see section 6c), and by spectrophotometric analysis.

(b) Preparation of malonaldehyde

Malonaldehyde was prepared by the method of Saslaw and Waravdekar (122). The colorless oblong hexagonal plates obtained by this method darkened above 185° without melting. The malonaldehyde was thus obtained in a pure form as the sodium salt (sodium salt of MA bis-bisulphite). Figure 1 shows the calibration curve for TBA-MA pigment. It may be seen that Beer's law was obeyed over the range 0-12 µg of sodium salt of MA-bis-bisulphite.

(c) Identification of the TBA-positive material

Paper chromatography:

The TBA-positive material which reacted with 2-thiobarbituric acid to give a reddish-orange pigment, was separated as the chromogen by extraction with a 1:1 mixture of isoamyl alcohol and hydrochloric acid. This extraction

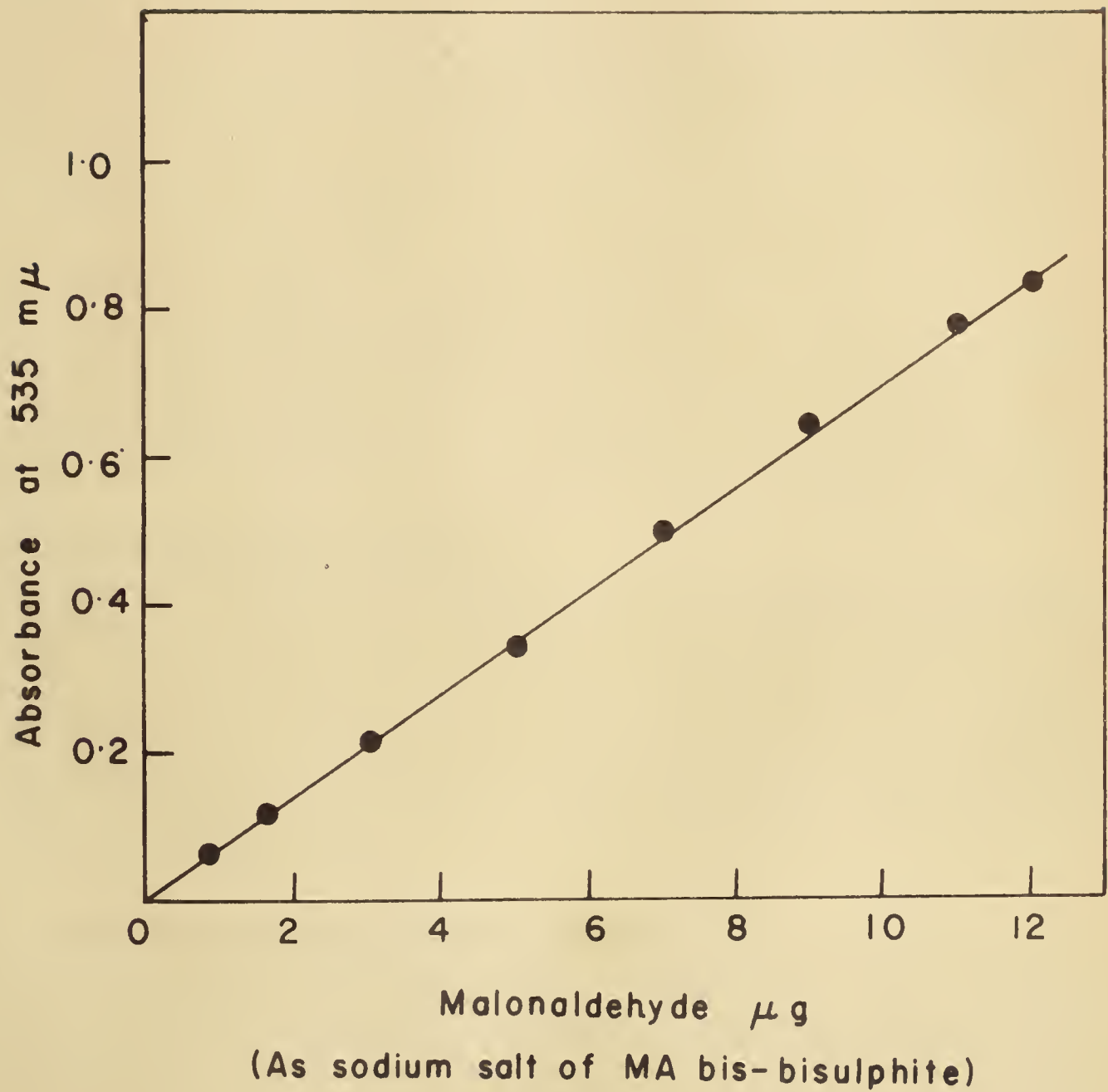


Figure 1
Relationship Between Concentration of Malonaldehyde
and Optical Density

was performed on the clear supernatant which is obtained after the stage when TBA reactants are treated with chloroform. 200 λ of such extracts were spotted on Schleicher Schull paper, No. 595. 20 x 20 cm. and 23 x 60 cm. sheets were used for ascending and descending chromatography, respectively. 200 λ of pure MA-TBA pigment, produced by reacting pure MA with thiobarbituric acid, was run on the same sheets.

Kitabchi and associates (16) reported that the pigments formed by the reaction of TBA with TBA-positive tissue components and with MA had identical R_F values in each of two solvent mixtures (pyridine-butanol- H_2O , 3:2:1.5; and 85% ethanol. In the studies described here, both these solvent systems were used, in addition to the ones noted below:

Butyric acid:Butanol: H_2O	2:2:1
95% Ethanol:Ammonia: H_2O	90:5:5
95% Ethanol: NH_4OH	100:1
Amyl alcohol:Acetic acid: H_2O	4:1:5
Isobutyric saturated with H_2O	
Butanol:Acetic acid: H_2O	4:1:5
Ether:Acetic acid: H_2O	13:3:1

Spectrophotometric studies:

Kohn and Liversedge (116) were the first to demonstrate the presence of a substrate in normal liver and other tissues, which when incubated aerobically produced pink color with TBA with an absorption maxima between 525 and 530 $m\mu$. Subsequent studies by other workers indicated that the TBA-positive material is malonaldehyde. The absorption spectra of the pigments obtained from tissue TBA-positive material, tissue lipid extract TBA-positive material, pure MA, etc., were studied.

7. Estimation of Polyunsaturated Fatty Acids

Although the alkali isomerization technique is quantitative for the estimation of the cis isomers of polyunsaturated fatty acids, it measures a fraction of the trans isomers. J. MacGee (121) reported a method for determining the total amount of polyunsaturated fatty acids by enzymic oxidation. This method was applied to the tissues in the present study.

Reagents were prepared as reported in this paper.

(1) 1M borate buffer, pH 9.0.

(2) 0.5N KOH in alcohol.

(3) Lipoxidase enzyme was obtained from Mann Research Laboratories, Inc., New York, N. Y. The stock lipoxidase solution (1 mg/ml) was never kept for more than 10 days in the freezer. The working solution was of 0.2 mg/ml concentration.

Procedure used in this laboratory:

One ml. of tissue homogenate in 0.05 M Tris, pH 7.4, was placed in a 50 ml. volumetric flask. To this was added 1 ml. of 0.5N alcoholic KOH and the flask placed in the dark for 24 hours, after which the excess KOH was neutralized by 1 ml. of 0.5N HCl. Ten mls. of 1 M borate buffer were then added and volume made up to 50 ml. with demineralized water, giving a solution of pH 9 and 0.2 M with respect to borate buffer.

After thorough mixing, the turbid samples from brain homogenates (heart homogenates gave clear solutions which did not need any centrifugation), were centrifuged. Two 3-ml. aliquots from each sample were placed in small tubes. 0.1 ml. of active enzyme was added to one tube, and 0.1 ml. of heated (for 5 mins., at 100°C) enzyme to the other. Both were mixed by inversion using parafilm as a stopper.

After 30 minutes of the enzymic action at room temperature, the optical density of the samples were read in the Beckman DU Spectrophotometer at 235 mμ. The samples with the heated enzyme served as blanks.

The same procedure was followed in estimating the PFA content of incubated tissue homogenates, of sub-cellular fractions and of the tissue lipid extracts.

MacGee calculated the PFA values as percent of the total oil sample. In this study, the results were expressed as mg PFA per gm of tissue or per whole organ weight in gm.

$$\text{mg PFA per gm of tissue} = A \times 0.6605 \times \frac{1000}{W}$$

where A = difference in absorbance between the blank and the sample;

W = mg. of tissue per ml.

1000 = conversion to mg. PFA per gm. of tissue.

$$\text{Source of factor } 0.6605 = \frac{3.1}{3.0} \times \frac{1}{78.2} \times 3 \times \frac{50}{3}$$

where

$$\frac{3.1}{3.0} = \text{dilution factor, 3 ml. of sample plus 0.1 ml. of enzyme}$$

78.2 = specific extinction coefficient (absorbance of 1 gm. of PFA per liter in 1-cm. light path at 235 $m\mu$)

$$3 \times \frac{50}{3} = \text{total sample volume.}$$

In order to check the validity of the assay procedure, the effects of varying several parameters of the system were examined. Thus the volume of KOH and the time during which the tissue preparation was treated with KOH were varied. In addition, since the lipid extracts were in ethanol, the effect of adding ethanol to samples of tissue homogenates was investigated. The results are summarized in Table I. The results indicate that (i) the saponification time in the range of 24-48 hours did not affect the PFA value. However, saponification time of 72 hours showed a slight decrease in the PFA value. (ii) Varying the KOH volume from 1 to 2 mls. did not affect the amount of PFA, and (iii) the addition of an extra ml. of ethanol did not produce any inhibitory effect on the enzyme action.

8. Estimation of Diene Concentration

The conjugated double bond system of unsaturated fatty acids have a strong absorption at 232.5 $m\mu$. This property has provided the basis of a convenient method for the quantitative estimation of these compounds. Maier and Tappel (123) applied this property of the conjugated double bonds to estimate them quantitatively in pure unsaturated fatty acid peroxides. This method could not be applied to tissue homogenates directly, because of interference by other tissue constituents. However, it was applied to their lipid extracts, and the results thus obtained were correlated with estimates of total PFA content and the estimates of the concentration of hydroperoxides and their breakdown products (see Results).

The lipid extracts in methanol were diluted 1:100 with methanol and their absorption at 232.5 $m\mu$, measured in a Beckman Spectrophotometer, Model DU, with methanol as blank.

TABLE I

Effect of Saponification Time and Saponification

Conditions on PFA Estimation

Time of Saponifi- cation in Hours	Additions	Mg. PFA per gm. of Tissue	
		Whole Homogenate ¹	Lipid Extracts ²
24	2 mls. alcoholic KOH	7.7939	7.3976
24	1 ml. alcoholic KOH + 1 ml. ethanol	7.6618	7.0353
24	1 ml. alcoholic KOH	7.7278	7.0675
48	2 mls. alcoholic KOH	7.6618	7.4636
48	1 ml. alcoholic KOH + 1 ml. ethanol	7.7278	7.0674
72	2 mls. alcoholic KOH	7.3315	6.7371
72	2 mls. alcoholic KOH + 1 ml. ethanol	7.5297	6.8032

¹Normal rat brain homogenates were used in this study in concentration of 100 mg/ml.

²The volumes of the lipid extracts employed were the same as the volumes of the tissue homogenates from which they were prepared.

IV. RESULTS

Section A

Lipid Peroxidation in Tissue Homogenates

1. Factors influencing the peroxidation measurement

The thiobarbituric acid (TBA) test has been employed in numerous laboratories to estimate the extent of lipid peroxidation in animal tissue preparations (6,8,124). The test's popularity arises from the fact that it is not only the most sensitive one available but is also extremely quick and simple to perform. Although the reaction is not quantitative, it has been felt that the test provides a reasonably good estimate of the degree of peroxidation. However, many investigators have handled tissue in widely divergent ways prior to the application of the TBA test. The present study was undertaken to investigate the effect of both the tissue pretreatment and tissue history on the production of the TBA-chromophore.

a. Non-stoichiometry of the TBA reaction: Simultaneous estimations of TBA-positive material and polyunsaturated fatty acids (PFA) were carried out on brain and heart homogenates of differing concentrations. The results, which are summarized in Table II, emphasize the non-stoichiometry of the test. It is quite clear from these data that results from different experiments in which different tissue concentrations were used cannot be compared directly.

However, in a large number of assays on tissue homogenates of fixed concentration, reproducible TBA-positive values were obtained, provided that the tissues were removed from animals of the same age, and which had been maintained on the same diet. It is evident that in comparative studies, the concentration of the tissue homogenates must be kept constant.

The PFA analyses tended to show fluctuations with different tissue concentrations in a manner somewhat similar to that noted for TBA color production (Table III). When the substrate concentration was too great (200 mg/ml for brain) the lipoxidase enzyme effect seemed to be inhibited. Perhaps lipoxidase behaves similar to catalase, which is gradually destroyed by excess substrate.

TABLE II
Effect of Tissue¹ Concentration on the Production of
the TBA-Chromogen in the Thiobarbituric Acid Test

Tissue Homogenate Concentration ³ mg/ml	Absorbance ² at 535 mμ per x mg/ml			
	Brain		Heart	
	Before Incubation (0 time)	After Incubation (90 minutes)	Before Incubation (0 time)	After Incubation (90 minutes)
200	0.202	0.446	-	-
150	0.184	0.746	-	-
100	0.208	0.668	0.092	0.280
50	0.224	0.544	0.074	0.218
25	0.234	0.384	0.060	0.144
12.5	0.184	0.286	0.040	0.098

¹Rats used were about 4 months old.

²Results are mean of 4 experiments.

³Five rat brains were pooled for each experiment, homogenized in Tris buffer, pH 7.4, to give a concentration of 200 mg/ml. Aliquots from this were then taken and diluted with the buffer to give the required concentrations. Similar procedure was followed with the rat hearts.

TABLE III
Effect of Tissue¹ Concentration on PFA Estimation

Concentration ³ used for assay mg/ml	Mg PFA ² per gm. of Tissue			
	Brain		Heart	
	Before Incubation	After 180 min. Incubation	Before Incubation	After 180 min. Incubation
200	3.5336	2.8732		
100	9.2470	8.1242	8.2562	8.2562
50	9.7754	8.6525	11.2285	11.2285
25	9.7754	8.5865	11.7040	11.7040
12.5	10.7264	9.2470	12.3117	12.0090

¹Rats used were about 4 months old.

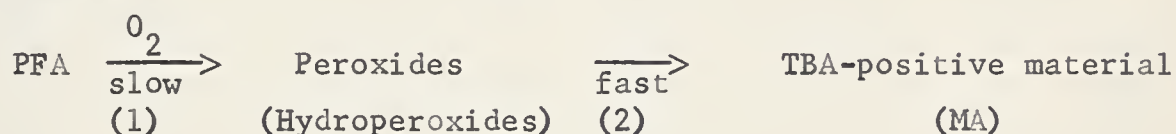
²Results are mean of 4 experiments.

³Five rat brains were pooled for each experiment, homogenized in Tris buffer, pH 7.4, to give a concentration of 200 mg/ml. Aliquots from this were then taken and diluted with the buffer to give the required concentrations. Similar procedure was followed with the rat hearts.

The values for PFA content per gm. of tissue obtained by using the tissue concentration range from 100 to 25 mg/ml, gave fairly constant results, in the case of brain. However, in the case of heart, either 25 or 50 mg/ml concentration was used.

b. Effect of incubation time on peroxidation: During the aerobic incubation of rat brain homogenate, there was a progressive increase in the concentration of TBA-positive material (peroxidation end products) during the first 180 minutes at least. At the same time there was a small, but definite decrease in the PFA level of the homogenate. These findings are illustrated graphically in Figure 2. When incubation was continued for more prolonged periods of time, a slight decrease in the amount of TBA-positive material present was noted (see Table IV).

The rate of formation of TBA-positive material which gives a chromogen with thiobarbituric acid, is linear for 180 minutes but falls off on prolonged incubation. Although the loss of PFA and the increase in TBA-positive material during incubation are related to each other, no quantitative evaluation can be made at this stage. It is known that PFA oxidation proceeds as



It is not clear from the incubation studies whether this process catalyzes the stage number (1) or stage number (2) or both. Perhaps during incubation, one stage of the above reaction sequence is influenced more than the other.

Conclusion: Since the rate of formation of TBA-positive material is linear with respect to time for at least 180 minutes, one can choose any incubation time period, of 180 minutes or less.

c. Effect of temperature on peroxidation: Heating the brain homogenate at 100°C for 10 minutes, produced a large amount of TBA-positive material (Table V). However, the heated tissue homogenate and the supernatant of the heated tissue homogenate showed responses similar to the unheated homogenate during a subsequent incubation period of 120 minutes.

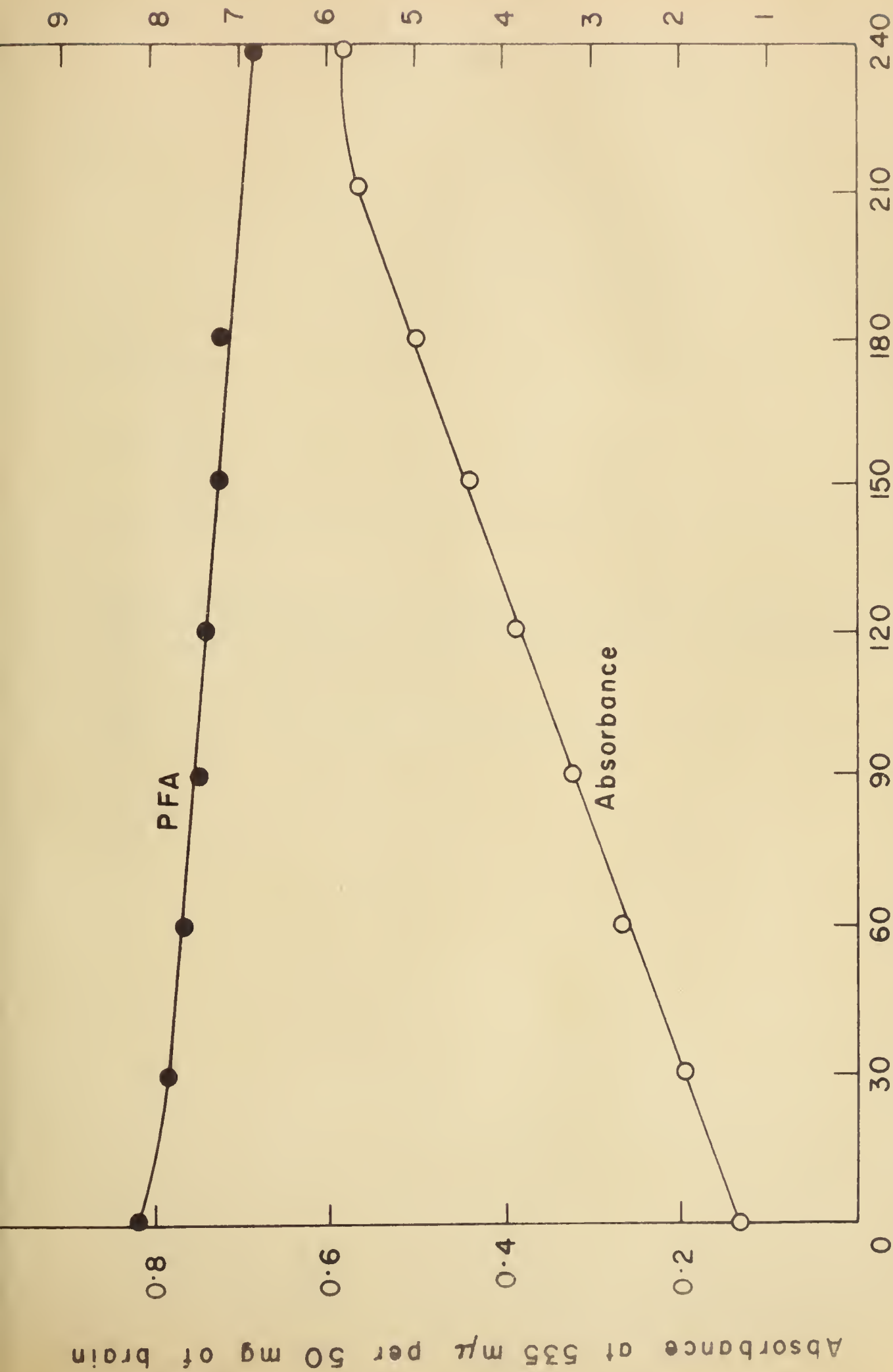


Figure 2

Increase in TBA-Positive Material and Decrease in PFA Level During
Aerobic Incubation of Rat Brain Homogenates

TABLE IV
Effect of Incubation Time on the
Production of TBA-Positive Material¹

Hours of Incubation at 37°C	0	1	2	3	6	24	48	72
Absorbance at 535 mμ ²	0.200	0.500	0.800	1.080	1.440	1.248	1.152	1.140

¹Brain tissue homogenates (50 mg/ml) in 0.05 M Tris buffer, pH 7.4, were incubated in a Dubnoff metabolic shaker at 37°C. The rats used in this study were about 4 months old.

²Absorbance is per 100 mg. of brain tissue.

TABLE V
Peroxidation in Heated Brain¹ Tissue Homogenates

Additions	Absorbance ² at 535 mμ per 50 mg. of tissue					
	Unincubated (0 time)			Incubated for 120 min.		
	Without Heating	With Heating ^a	With Heating ^b	Without Heating	With Heating ^a	With Heating ^b
None	0.120	0.320	0.290	0.430	0.448	0.376
³ Iron 10 ⁻³ M	0.304	0.460	0.416	0.746	0.618	0.522
" 10 ⁻⁴ M	0.166	0.362	0.316	0.640	0.500	0.442
⁴ Vanadium 10 ⁻³ M	0.308	0.456	0.406	0.710	0.670	0.568
" 10 ⁻⁴ M	0.168	0.348	0.362	0.482	0.476	0.412
Ascorbic 10 ⁻³ M	0.124	0.324	0.354	0.682	0.716	0.636
" 10 ⁻⁴ M	0.134	0.332	0.346	0.472	0.482	0.442

¹Six month old normal rats were used.

^aCoagulated mass and the supernatant rehomogenized.

^bOnly the supernatant fluid obtained by centrifugation.

²Results are mean of 3 experiments.

³Added as ferrous-sulphate.

⁴Added as vanadyl-sulphate.

Most of the TBA-positive material appears in the supernatant fluid of the boiled homogenate, after centrifugation. This is apparent from the very slight difference in TBA-positive values obtained from the supernatant (b) and rehomogenized heated tissue (a) (Table V) both before and after incubation. The slightly higher TBA-positive values of the rehomogenized (a) material could be due to adsorption of the TBA-positive material on the coagulated protein mass. The effect of added metallic ions would be discussed under 2.d. of this section, "Cofactors and inhibitors of peroxidation."

Discussion: Heating brain tissue homogenate at 100°C for 10 minutes, caused a rapid accumulation of the TBA-positive material. Such a procedure would probably destroy all the tissue enzymes. However, the homogenates, heated in this way still produced further TBA-positive material on aerobic incubation at 37°C. This strongly suggests that peroxidation is a non-enzymic process.

There is a marked increase in TBA-positive material on heating initially but the final TBA-positive values appear to be almost the same in the unheated and heated incubated samples. This indicates that heat accelerates the initial peroxidation, however, the amount of peroxidation is the same after incubation, irrespective of whether heated or unheated homogenates are employed.

Conclusion: The above findings suggest that in vitro lipid peroxidation is a non-enzymic process.

d. Incubation medium: The results of the experiments designed to test the effects of the incubation medium in the formation of TBA-positive material are presented in Tables VI and VII. The three media examined in these studies were 0.15 M KCl, and phosphate and Tris buffer of pH 7.4.

Brain tissue from normal rats were used in this study. Lower TBA-positive values were obtained, with the whole homogenate, when incubations were carried out in 0.15 M KCl, than when either Tris or phosphate buffer of pH 7.4 was used. On the other hand, the TBA-positive values obtained in the phosphate buffer were higher than those obtained in the Tris buffer.

TABLE VI

Effect of Incubation Medium on the Production of TBA-Positive
Material and the PFA Content in Rat Brain Homogenates¹

Buffers ² :	Tris		Phosphate		KCl	
Incubation time in minutes:	0 time	180 min.	0 time	180 min.	0 time	180 min.
Absorbance ³ at 535 mμ per 100 mg. of brain tissue						
	0.140	0.790	0.176	1.040	0.108	0.568
PFA ³ per gm. of brain tissue						
	9.2801	8.9498	8.4214	7.3315	9.0819	8.8375

¹Six months old rats were used, with brain homogenate concentration
= 50 mg/ml.

²Tris - 0.05 M, pH 7.4.

Phosphate - 0.15 M, pH 7.4.

KCl - 0.15 M, pH 6.6.

³Results are mean of 3 experiments.

TABLE VII

Effect of Incubation Medium on TBA-Positive Material
Production and PFA Level in Rat Brain¹ Subcellular Particles

Buffers:	Tris		Phosphate		KCl	
Incubation time:	0 time	180 min.	0 time	180 min.	0 time	180 min.
Fraction	Absorbance at 535 mμ per 100 mg. of brain tissue					
Mitochondria	0.056	0.068	0.048	0.066	0.052	0.068
Microsomes	0.052	0.058	0.050	0.056	0.050	0.054
Supernatant	0.116	0.122	0.138	0.138	0.058	0.060
mg PFA per gm of brain tissue						
Mitochondria	0.7662	0.7266	0.4888	0.3038	0.3963	0.3303
Microsomes	0.2642	0.1321	0.2372	0.1321	0.1849	0.06605
Supernatant	-	-	-	-	-	-

¹Conditions similar to those of Table VI.

Maximum PFA level was detected in the Tris buffer, both before and after incubation of the whole homogenate. At the subcellular level, the TBA-positive values in the mitochondria and the microsomes appeared to be the least affected in any of the three media.

Conclusion: Throughout these studies on lipid peroxidation, incubation medium of 0.05 M Tris buffer, pH 7.4, was used.

e. pH of the medium: The pH of the medium appeared to influence the formation of the TBA-positive material and also the disappearance of the polyunsaturated fatty acids (Tables VIII and IX).

Between pH's 7 and 8, the effect of slight changes in pH on the production of TBA-positive material appears to be minimal. This is also the range of pH in which the PFA level seems to be little affected.

Discussion: Most of the studies reported herein were carried out in 0.05 M Tris buffer, pH 7.4; as at about this pH the amount of TBA-positive material produced does not vary with slight change in pH, and since the detectable PFA content of the tissue is maximum. Moreover, pH 7.4 is to be favored, as it is also the pH of the living tissue. However, the differences noted in Tables VIII and IX cannot be ascribed to pH alone since four different buffer systems were utilized over the pH range used.

Conclusion: The results tend to indicate that around pH 7.4, the effect of pH on peroxidation of the PFA is minimum.

2. Studies relating to the mechanism of lipid peroxidation

Polyunsaturated fatty acids are known to autoxidize to produce hydroperoxides, which under different conditions may give rise to products containing carbonyl groups, their polymerized products, etc. One such product, demonstrated by many workers to be formed in tissues under physiological conditions, is malonaldehyde (12,13,14).

a. End products:

(i) Paper chromatography: Separation of the chromogen formed in the TBA test with the tissue TBA-positive material, was carried out (see Methods). The chromatograms are shown (Figures 3, 4, 5 and 6).

Two spots were obtained with the different solvents used, in both

TABLE VIII

Influence of pH on the Formation of TBA-Positive Material

pH	Absorbance at 535 mμ per 100 mg. of brain tissue ¹							
	Buffers:	Acetate		Phosphate		Tris		Borate
	Incubation time in minutes:	0	180	0	180	0	180	0 180
3.6		0.200	0.950					
4.0		0.234	0.940					
5.0		0.222	1.040					
6.0				0.216	1.060			
7.0				0.226	0.990			
7.2						0.188	0.800	
7.4				0.194	1.620	0.190	0.860	
8.0				0.230	0.920	0.186	0.750	
8.4						0.200	0.656	
9.0						0.196	0.500	0.196 0.356
11.0				0.194	0.264			
12.0				0.268	0.270			

¹Three month old normal rats were used in this study. The brain tissue homogenate concentration was 50 mg/ml.

TABLE IX

Influence of pH on PFA Levels in Brain¹ Tissue Homogenates

		mg PFA per gm. of brain wet weight							
Buffers:		Acetate		Phosphate		Tris		Borate	
pH	Incubation time in minutes:	0	180	0	180	0	180	0	180
3.6		n.d. ²	n.d.						
4.0		7.1334	5.3897						
5.0		8.9168	6.4465						
6.0				7.9260	5.6539				
7.0				8.3223	6.0766				
7.2						8.5205	8.5205		
7.4						8.5865	7.9921		
8.0				8.3884	8.0977				
						8.5205	7.926		
8.4						8.3884	8.2563		
9.0						9.0489	9.1149	8.3884	6.1427
11.0				9.1242	9.2470				
12.0				9.9828	9.9075				

¹Brains from 3-month old rats were used at an homogenate concentration of 50 mg/ml.

²Not detected.

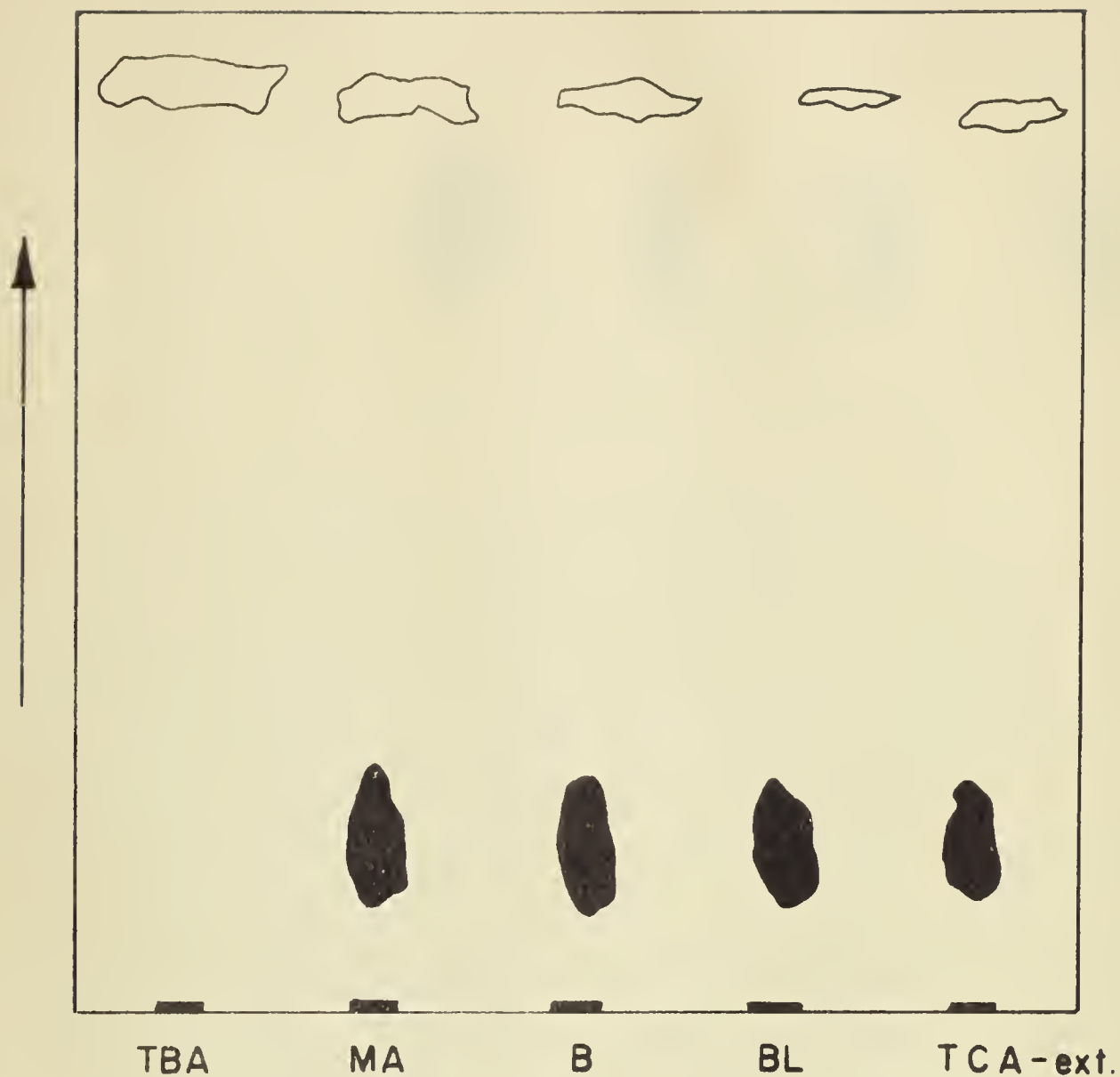


Figure 3

An ascending chromatogram of the pigments with the mobile phase solvent as ether:acetic:H₂O (13:3:1). The pigments were obtained by the interaction of TBA² with -

MA = Malonaldehyde

B = TBA-positive material from brain homogenate

BL - " " " " " lipids

TCA-ext = " " " " " homogenate cold TCA-extract



Figure 4

An ascending chromatogram of the pigments with the mobile phase solvent as pyridine:butanol:H₂O (3:2:1.5). The pigments were obtained by the interaction of TBA with -

MA = Malonaldehyde

B = TBA-positive material from brain homogenate

BL = " " " " " lipids

TCA-ext = " " " " " homogenate cold

TCA-extract

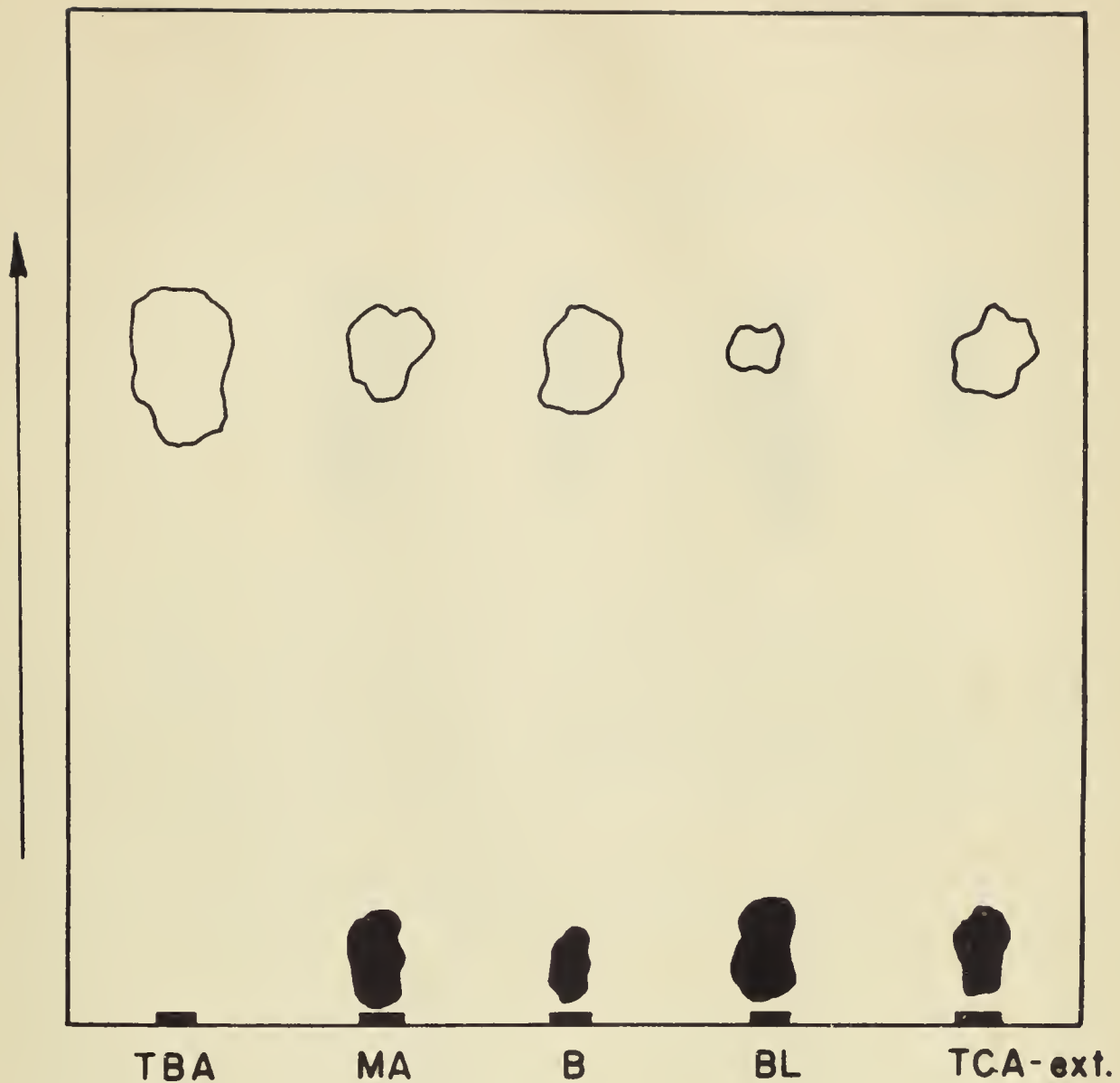


Figure 5

An ascending chromatogram of the pigments with the mobile phase as 85% ethanol. The pigments were obtained by the interaction of TBA with -

MA = Malonaldehyde

B = TBA-positive material from brain homogenate

BL = " " " " " lipids

TCA-ext = " " " " " homogenate cold
TCA-extract

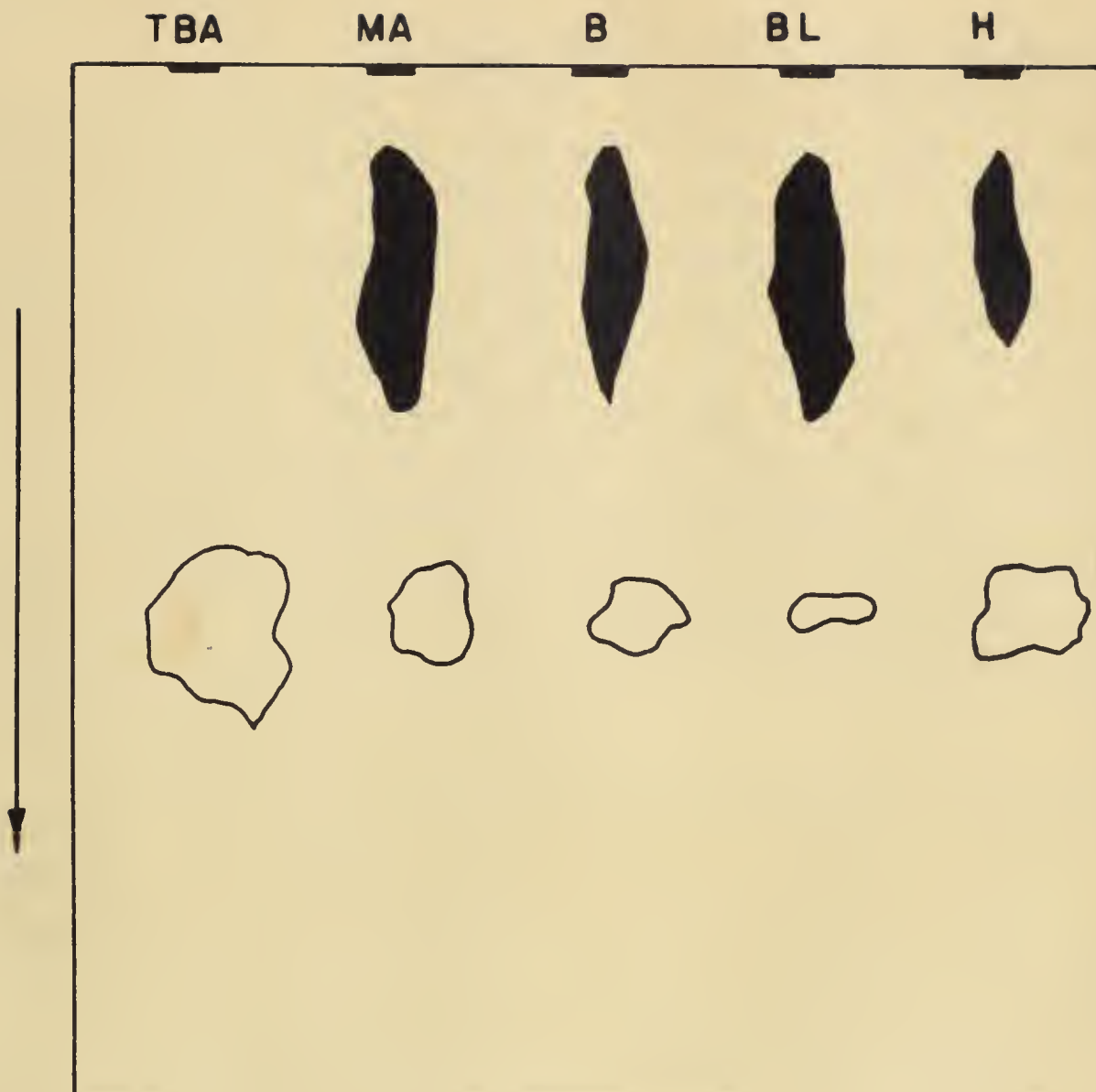


Figure 6

A descending chromatogram of the pigments with the mobile phase as 85% ethanol. The pigments were obtained by the interaction of TBA with -

MA = Malonaldehyde

B = TBA-positive material from brain homogenate

BL = " " " " lipids

H = " " " " heart homogenate

ascending and descending chromatograms. Spot number 1 with low R_F value was identified as the chromogen formed by the interaction of malonaldehyde with TBA. The second spot with higher R_F value was identified as the one obtained by interaction of TBA with the mobile solvent phase used. No other chromogen was separated.

(ii) Spectrophotometric studies: When the TBA-chromogen, obtained by the TBA test on brain or heart tissue homogenates, was studied spectrophotometrically, it gave a main peak at 535 $m\mu$ and a small peak at 450 $m\mu$ (Table X). The 535 $m\mu$ absorption corresponds to the MA-TBA (malonaldehyde-thiobarbituric acid complex) pigment, as shown by studying the MA-TBA pigment obtained by reacting pure MA with TBA, under the conditions of the TBA test. The pigment obtained with pure MA did not give any peak at 450 $m\mu$, but the pigment obtained from pure linolenic acid peroxidation products exhibited a peak at 450 $m\mu$, and 535 $m\mu$, like the tissue homogenates.

Discussion: The results of both chromatographic and spectrophotometric studies indicated that the TBA-positive material formed in tissues, was malonaldehyde. Absorption spectra of chromogens formed from TBA-positive component of tissue is not identical with that formed from malonaldehyde. The former has a peak at 450 $m\mu$ also.

The small peak obtained at 450 $m\mu$ with the tissue homogenate TBA-chromogen is not obtained with pure MA-TBA chromogen. It appears that the absorption peak at 450 $m\mu$ might be caused by a peroxidic product from the tissue or by the interaction of a tissue component with TBA, which is not separated from the main chromogen by our chromatographic procedures.

The lipids extracted from the tissue homogenates also produced the TBA-chromogen with absorption peaks at 535 $m\mu$ and 450 $m\mu$, thus providing suggestive evidence that the 450 $m\mu$ absorption is caused by a peroxidic product from the tissue lipids. Furthermore, pure linolenic acid also gave an absorption peak at 450 $m\mu$. The absorption at 450 $m\mu$ decreases slightly, while that at 535 $m\mu$ increases a little, when the chromogen is left at room temperature for 24 hours. It is possible, therefore, that this peroxidation product, with absorption at 450 $m\mu$ might be a precursor of malonaldehyde.

TABLE X
Spectral Study of the TBA-Chromogens

Wave-length	Absorbance					
	TBA	MA-TBA pigment	Linolenic TBA pigment	Brain ¹ -TBA pigment	Brain-Lipid ² TBA pigment	Brain-cold TCA ³ extract TBA pigment
400	0.125	0.057	0.230	0.320	0.250	0.115
425	0.034	0.040	0.285	0.340	0.600	0.030
450	0.035	0.105	<u>0.560</u>	<u>0.550</u>	<u>1.000</u>	0.050
475	0.022	0.280	0.500	0.300	0.450	0.100
500	0.020	0.670	1.100	0.200	0.370	0.240
530	0.020	1.800	>2.000	0.990	0.950	0.660
535	<u>0.020</u>	<u>1.950</u>	<u>>>2.000</u>	<u>1.050</u>	<u>1.100</u>	<u>0.730</u>
538	0.017	1.400	2.000	1.000	0.950	0.670
545	0.015	0.112	0.270	0.200	0.050	0.080
550	0.010	0.100	0.265	0.198	0.040	0.070

¹Pigment obtained by interaction of TBA-positive material of brain tissue after 180 minutes incubation; with TBA.

²Lipid extract of 1.

³Cold TCA-extract obtained after precipitation of lipid extract (2) from the brain homogenate (1).

(iii) Studies with possible precursors: Kaufmann and Vogelmann in 1959 (125) reported the separation of heptanal, hexanal and short chain aldehydes from autoxidized linseed oil. In the present study, several aldehydic compounds, which on a theoretical basis, might be formed by the oxidative breakdown of brain or heart PFA, were obtained in pure form. All were examined in the thiobarbituric acid test, both before and after incubation at 37°C, and the absorption spectra of the reaction mixtures were determined. None of them gave rise to a chromogen having a significant absorption at either 535 or 450 mμ (Table XI). Only MA gave a significant color with TBA and the MA-TBA chromogen was found to exhibit an absorption at 535 mμ that was about 20 times greater than that of the chromogen formed from equimolar quantities of any of the other aldehydes.

Conclusion:

(1) Malonaldehyde is the major product of lipid peroxidation in tissues. Another peroxidation product, the identity of which is unknown, is formed in minute amounts.

(2) It appears that with the exception of malonaldehyde, none of the aldehydes studied can contribute to any significant extent to the formation of chromogen in the TBA-test, even if they are present in the tissues.

b. Polyunsaturated fatty acid levels: During aerobic incubation, the polyunsaturated fatty acid content of brain tissue homogenates gradually decreased, as the concentration of TBA-positive material increased (Table XII and Figure 2).

Discussion: There is a direct effect on the PFA level during peroxidation. It is difficult, however, at this stage to establish any quantitative relationship between the levels of PFA and peroxides and their breakdown products.

c. Lipid extracts: Lipids were extracted from the brain tissue homogenates, both before and after incubation. When TBA reaction was performed on the lipid extracts, before incubation, very high TBA-positive values were obtained as compared to TBA-positive values from the whole homogenates of the same concentration (Table XII). Similar results were observed after incubation. But the TBA-positive values from the lipid

TABLE XI

Study of TBA Test with Several Aldehydic Compounds

Number of carbon atoms	Aldehyde	Formula	Absorbance at 535 mμ per 10 μmoles	
			Before incubation	After 180 min. incubation
C ₃	Malonaldehyde (bis-bisulphite)	$\begin{array}{c} \text{ONa} \\ \diagup \\ \text{CH} - \text{HSO}_3 \\ \\ \text{CH}_2 - \text{CHO} \end{array}$	1.060	1.040
C ₅	Glutaric dialdehyde	$(\text{CH}_2)_3 \begin{array}{l} \diagup \text{CHO} \\ \diagdown \text{CHO} \end{array}$	0.062	0.058
C ₆	Valeraldehyde-2- methyl	$\text{CH}_3(\text{CH}_2)_2 \begin{array}{c} \text{CH} - \text{CHO} \\ \\ \text{CH}_3 \end{array}$	0.036	0.028
C ₈	Octyl aldehyde	$\text{CH}_3(\text{CH}_2)_6\text{CHO}$	0.064	0.080
C ₉	Nonyl aldehyde	$\text{CH}_3(\text{CH}_2)_7\text{CHO}$	0.082	0.096
C ₁₀	Decyl aldehyde	$\text{CH}_3(\text{CH}_2)_8\text{CHO}$	0.072	0.076
C ₁₀	Sebacic acid (half aldehyde)	$(\text{CH}_2)_8 \begin{array}{l} \diagup \text{COOH} \\ \diagdown \text{CHO} \end{array}$	0.076	0.082
C ₁₁	n-undecyclic	$\text{CH}_3(\text{CH}_2)_9\text{CHO}$	0.082	0.072
C ₁₁	n-undecyclenic	$\text{CH}_3\text{CH}=\text{CH}(\text{CH}_2)_7\text{COOH}$	0.078	0.058
C ₁₂	Dodecyl	$\text{CH}_3(\text{CH}_2)_{10}\text{CHO}$	0.064	0.046
C ₁₂	Suberic dialdehyde tetramethyl acetal	$\begin{array}{c} \text{OCH}_3 \\ \diagup \\ \text{CH} - \text{OCH}_3 \\ \\ \text{CH}_2 \\ \diagdown \\ \text{CH} - \text{OCH}_3 \\ \\ \text{OCH}_3 \end{array}$	0.064	0.060
C ₁₄	Tetradecyl	$\text{CH}_3(\text{CH}_2)_{12}\text{CHO}$	0.062	0.066
C ₁₆	Hexadecyl	$\text{CH}_3(\text{CH}_2)_{14}\text{CHO}$	0.052	0.070
C ₁₈	Octadecyl	$\text{CH}_3(\text{CH}_2)_{16}\text{CHO}$	0.038	0.054

TABLE XII

Changes¹ in the Levels of TBA-Positive Material and PFA
During in vitro Incubation of Rat Brain² Homogenates³ and of
Lipid Extracts⁴ Prepared Therefrom

Fraction	Hours of Incubation			
	0	3	6	72
mg PFA per gm. of brain tissue				
Whole homogenate	9.8	8.8	8.1	6.5
Lipid extract ^b	9.5	8.7	8.2	7.0
Absorbance at 535 mμ per 100 mg. of tissue				
Whole homogenate	0.200	1.080	1.440	1.140
Lipid extract ^a	0.760	0.820	0.760	0.200
Cold TCA extract ^b	0.140	0.800	1.260	1.000

¹Results are from six sets of experiments.

²Rats were about 4 months old.

³Homogenate concentration 50 mg/ml.

⁴Lipid extraction method (see Methods).

^aLipid extract is the lipid material extracted from the precipitate noted in b.

^bCold TCA-extract is the soluble material after precipitation of protein and lipid with TCA.

extracts obtained before and after incubation, did not differ much. The cold trichloroacetic acid (TCA) extract also showed the presence of TBA-positive material. Maximum levels of TBA-positive material were reached after 6 hours of incubation, in both the whole homogenate and in the cold TCA extract, while the TBA-positive material in the lipid extracts remained relatively constant up to 6 hours of incubation.

The cold TCA extract would be expected to contain most of the MA, which is readily water soluble. The pigment separated from this after reaction with TBA, gave an absorption peak at 535 m μ but no peak at 450 m μ such as that observed with the product of the reaction between TBA and the TBA-positive material formed in tissue homogenates and their lipid extracts.

Thus it is apparent from the results that the comparable TBA-positive value to the whole homogenate should be taken as the TBA-positive value of the lipid extract plus the TBA-positive value of its cold TCA-extract.

The PFA levels in both the whole homogenate and its lipid extract showed a progressive decrease with incubation time, but no significant difference was noted between the PFA content of the whole homogenate and its lipid extract at any one time.

Heart tissue showed low level of in vivo peroxidation products and very little susceptibility to peroxidation in vitro (Table XIII). However, the lipid extracts from the heart tissue homogenates, both before and after incubation showed responses similar to brain lipid extracts.

Discussion: The occurrence of an amount of TBA-positive material in the lipid extract in excess of that found in the whole homogenate was unexpected. The extraction must catalyze either the production or liberation of TBA-positive material, i.e. malonaldehyde.

The PFA level is not lowered significantly during lipid extraction, i.e. the PFA level in the whole homogenate and its lipid extract is practically the same both before and after aerobic incubation. Hence it is difficult to account for the presence of high levels of TBA-positive material in the lipid extracts. It is possible that the lipid extraction procedure, either catalyzes the production of TBA-positive material (MA) from its precursor or liberates MA from the particulate system, which

TABLE XIII

Changes¹ in the Levels of TBA-Positive Material and PFA
During in vitro Incubation of Heart² Tissue Homogenates³
and of Lipid Extracts Prepared Therefrom

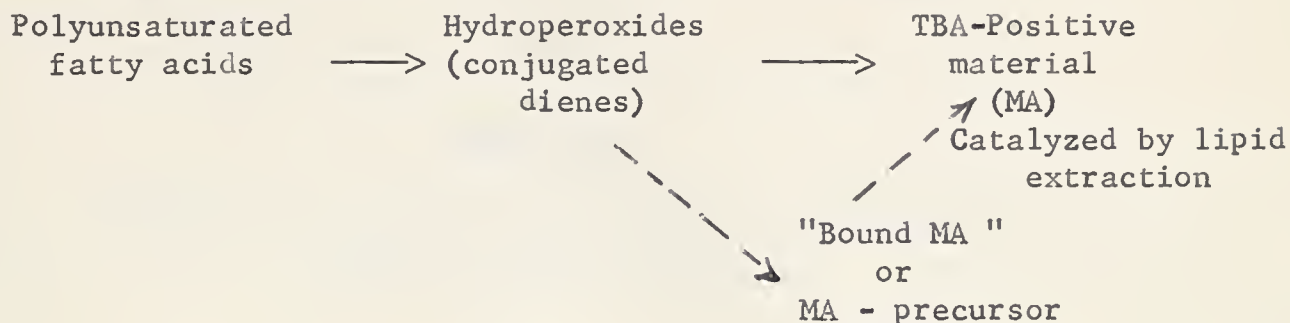
Fraction	Incubation time	
	0 time	180 min.
Absorbance at 535 mμ per 100 mg of heart tissue		
Whole homogenate	0.112	0.124
Lipid extract	0.460	0.404
mg PFA per gm. of heart tissue		
Whole homogenate	10.1188	10.0856
Lipid extract	10.0088	10.0856

¹Results are from 4 sets of experiments.

²Four-month old rats.

³Homogenate concentration 25 mg/ml.

ordinarily may not be accessible in the whole homogenate. Thus:



As there is no suitable method for estimating peroxides in the tissue homogenates directly, it is difficult to say whether the peroxides are destroyed during the lipid extraction or whether there is a precursor(s) of TBA-positive material (MA) which is released from some bound form during this extraction, giving rise to the high TBA-positive values.

The values shown in Table XIV indicate some of the changes occurring during aerobic incubation. Immediately after excision, there are products of lipid peroxidation present in the brain. Both the whole homogenate and the lipid extract derived from it, contained TBA-positive material.

The absorption at 232.5 mμ is indicative of the presence of material containing conjugated systems of double bonds - the conjugated diene hydroperoxides (55,123). After incubation, the amount of TBA-positive material in the whole homogenate increased, the TBA-positive material in the lipid extract remained relatively constant, the conjugated hydroperoxide level decreased. The concentration of PFA was slightly less after incubation.

The TBA-positive material in the lipid extract has not been identified. Malonaldehyde could not be removed from the lipid by acid washing. However, the red pigment obtained could not be differentiated from the MA-TBA chromogen by chromatography using different solvent systems (see Figures 3, 4, 5 and 6), and its absorption spectrum was quite similar to that of the pigment produced from the whole homogenates. Until this pigment(s) is identified, it might be referred to as "Bound malonaldehyde".

d. Cofactors and inhibitors of peroxidation: The addition of iron or vanadium or ascorbic acid to a brain tissue homogenate caused a rapid increase in TBA-positive values, both at zero time and after incubation (Tables XV and XVI; and Figures 7 and 8).

TABLE XIV

Changes in Rat Brain Homogenates¹ and Its Lipid
Extracts after Incubation

Incubation time:	0 time	120 minutes
TBA-positive material values:		
(absorbance at 535 mμ)		
(a) Whole homogenate	0.202	0.742
(b) Lipid extract	0.680	0.710
(c) Cold TCA extract	0.064	0.656
Conjugated-diene value:		
(absorbance at 232.5 mμ)		
(a) Lipid extract	0.203	0.164
PFA level (in mgs.):		
(a) Whole homogenate	1.858	1.694

¹All values are for 200 mg. tissue taken from animals 15 days of age.

TABLE XV

Influence of Various Metal Ions on the Rate of Lipid Peroxidation in Rat Brain Homogenates

Compound ¹	Valence	Concentration x 10 ⁻³ Molar	TBA Units as percent of unincubated control		Percent change after incubation over the incubated control
			0 time	120 min.	
Whole homogenate			100	306	0
<u>Metallic ions:</u> ²					
[*] W _{hom} + vanadium	4	1	261(4) ³	500	+65
" "	4	0.1	130(4)	380	+25
" iron	2	1	231(6)	491	+62
" "	2	0.1	181(6)	412	+35
" "	3	1	196(4)	406	+33
" "	3	0.1	140(4)	353	+15
" cesium	1	1	101(2)	284	- 4
" "	1	0.1	114(2)	269	-12
" cerium	3	1	96(2)	205	-33
" "	3	0.1	107(2)	280	- 8
" "	4	1	121(2)	223	-27
" "	4	0.1	100(2)	293	- 4
" cobalt	3	1	101(3)	117	-66
" "	3	0.1	109(3)	298	-36
" copper	2	1	84(1)	157 ⁴	-50
" manganese	2	1	96(3)	99	-69
" "	2	0.1	99(3)	111	-65

¹Additions made to homogenate before taking zero time aliquots.

²The metallic ions used were in the form: vanadyl-sulphate, ferrous-sulphate, ferric-sulphate, cerous sulphate, ceric sulphate, cobalt chloride, copper-sulphate, manganese chloride, and cesium-chloride.

³Brackets indicate number of experiments.

⁴TBA units after 180 minute incubation.

^{*}W_{hom} = whole homogenate.

TABLE XVI

Influence of Various Compounds on the Rate of Lipid Peroxidation in Rat Brain Homogenates

Compound ¹	Concentration x 10 ⁻³ M	TBA units as per cent of unincubated control		Percent change after incubation over the incubated control
		0 time	120 min.	
Whole homogenate		100	306	
<u>Antioxidants:</u>				
W _{hom} * + Methylene blue	1	63(2) ²	84	-74
" " "	0.1	96(2)	180	-42
" Diphenyl-p-phenylene diamine (DPPD)	1	45(2)	49	-86
" α-tocopherol	1	60(2)	60	-82
" Ascorbic acid	10	99(6)	606	+100
" "	1	110(6)	494	+ 63
" "	0.1	120(6)	377	+ 24
" Ethylene diamine tetra acetate, Na (EDTA)	0.1	80(2)	80	- 75

¹Additions made to homogenates before taking 0 time aliquots.

²Brackets indicate number of experiments.

*W_{hom} = whole homogenate.

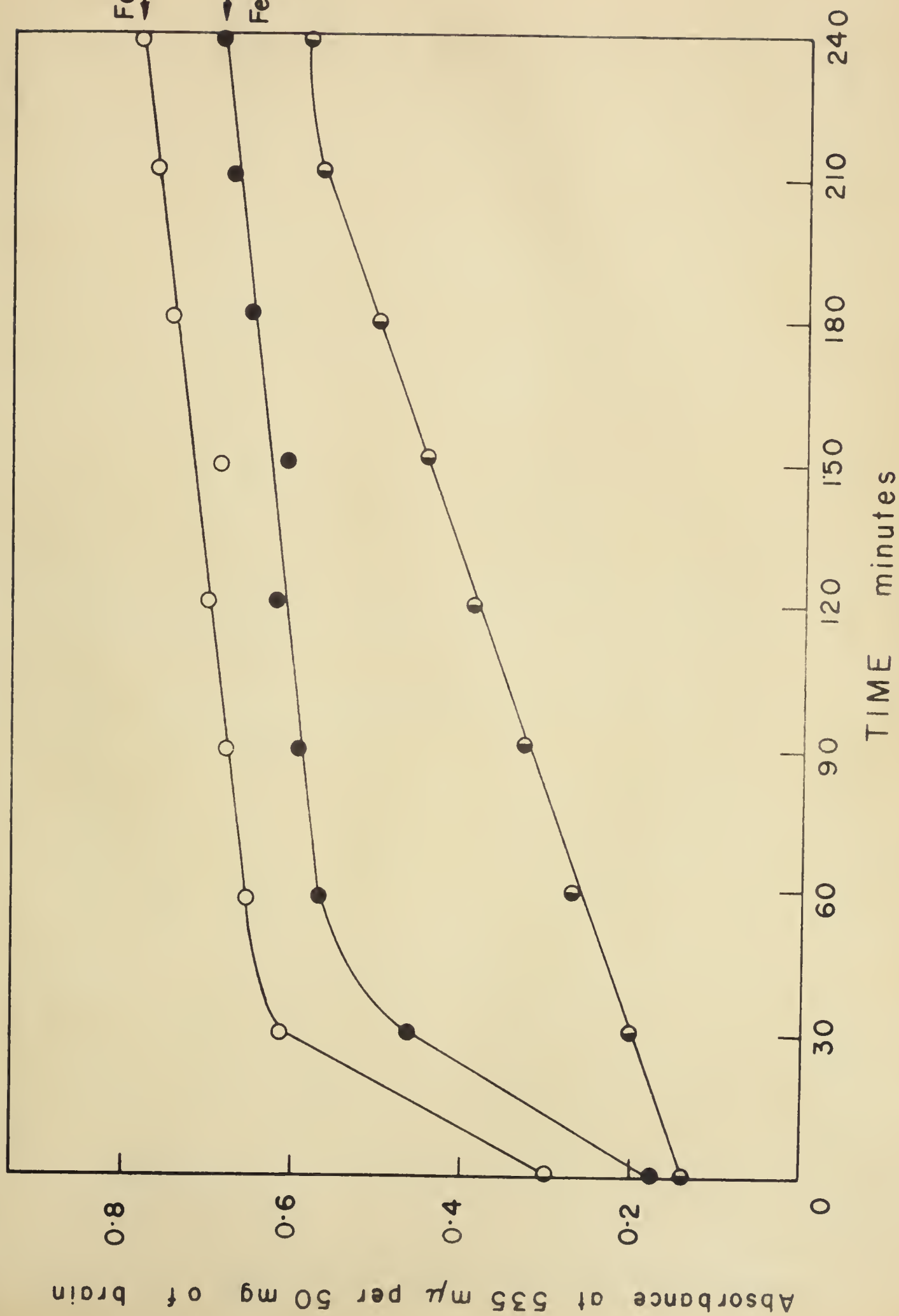


Figure 7
Increase in TBA-Positive Material During Aerobic Incubation of Rat Brain Homogenates,
With and without Added Fe^{++} Ions
(Half-open circles represent only whole homogenate.)

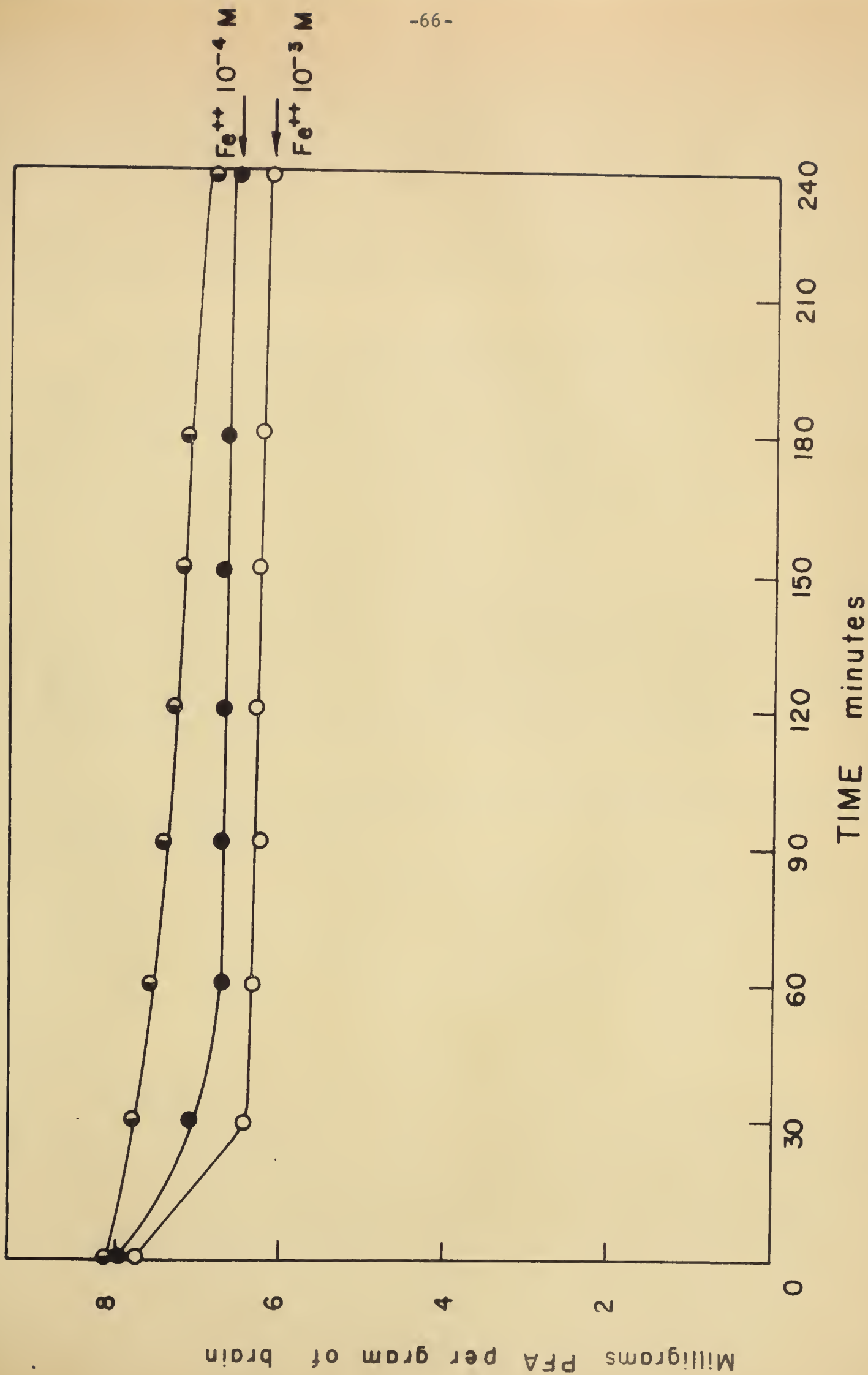


Figure 8

Decrease in PFA Levels During Aerobic Incubation of Rat Brain Homogenate, With and Without Added Fe^{++} Ions. (Half-open circles represent only whole homogenate)

Iron stimulated increase in TBA-positive material, was accompanied by a drop in the PFA level (Figures 7 and 8) on incubation. However, the drop in PFA level on addition of iron at zero time was insignificant (Figure 8) but the gain in TBA-positive material was quite significant (Figure 7). This indicates that the metallic ions catalyze the breakdown of peroxides or MA-precursor(s), and to a lesser degree the breakdown of the PFA.

The common cations, sodium, potassium, calcium and magnesium, had no effect on TBA-positive values in either brain or heart homogenates. Certain other metals like manganese and copper inhibited peroxidation. Chelating agents and antioxidants suppressed the in vitro peroxidation. This demonstrates that the oxidative reactions require metallic cofactors.

Similar effects were observed in the microsomal-supernatant fraction of both brain and heart (Table XVII).

Conclusion: The above findings demonstrate that in vitro lipid peroxidation is a metal catalyzed, non-enzymic (Section A.1.c.) process. It does seem reasonable to assume that the in vivo situation is somewhat similar. However, in the living animal, enzymic reactions concerned with numerous cellular functions may influence a tissue's rate of peroxidation.

3. Examination of sub-cellular fractions prepared by high speed centrifugation for lipid stability

The results of studies concerning lipid stability in sub-cellular fractions are presented in Tables XVIII and XIX. The sub-cellular fraction of brain which possessed the greatest relative amount of peroxides was the microsomal-supernatant fraction, - that portion remaining after sedimentation of nuclei and mitochondria from tissue homogenates. The heart sub-cellular fractions like the whole heart homogenate showed low levels of peroxidic products. This will be discussed further in Section B.

Discussion: Kitabchi et al. (16) have noted a somewhat similar occurrence in liver homogenates. Earlier Tappel and Zalkin (6,7) had reported that the mitochondria were a site of lipid peroxidation. It is not inconceivable that some TBA-positive material, arising from lipid peroxidation in other cellular compartments, could be released into the soluble cytoplasm.

TABLE XVII

Influence of Various Compounds on the Rate of Lipid Peroxidation
in Rat Brain Sub-Cellular Fraction-Microsomal Supernatant

Compound ¹	Valence	Concentra- tion x 10 ⁻³ M	TBA units as percent of unincubated micro- somal-supernatant		Percent change after incuba- tion over the incubated control
			0 time	120 min.	
Microsomal-super- natant (Mic/sup)			100	320	
Mic/sup. + vanadium	4	1	229	426	+33
" "	4	0.1	141	353	+10
" iron	2	1	300	412	+29
" "	2	0.1	135	406	+27
" cesium	1	1	85	303	- 5
" "	1	0.1	118	332	+ 3
" cerium	3	1	79	153	-52
" "	3	0.1	115	274	-14
" manganese	2	1	100	97	-70
" "	2	0.1	124	132	-59
" ascorbic acid		10	129	382	+20
" "		1	141	353	+10
" "		0.1	126	347	+ 8

¹Additions made to microsomal-supernatant fraction before taking zero time aliquots.

²Results are from 3 different experiments.

TABLE XVIII

Distribution and Formation of Lipid Peroxidation Products
in Sub-Cellular Fractions of Normal Rat¹ Heart² and Brain³

Sub-cellular fraction	Absorbance at 535 m μ per 100 mg. of tissue		
	Before incuba- tion	After 180 min. incubation	Percent increase during incubation
<u>Brain:</u>			
Mitochondria	0.068(4) ⁴	0.076(4)	12
Microsomes	0.052(4)	0.058(4)	12
Supernatant	0.110(4)	0.120(4)	9
Microsomal- supernatant	0.136(4)	0.444(4)	226
<u>Heart:</u>			
Mitochondria	0.088(4)	0.100(4)	14
Microsomes	0.084(4)	0.112(4)	33
Supernatant	0.108(4)	0.132(4)	20
Microsomal- supernatant	0.100(4)	0.120(4)	20

¹Three-month old normal rats were used.

²Heart homogenate concentration = 25 mg/ml.

³Brain homogenate concentration = 50 mg/ml.

⁴Brackets indicate the number of experiments.

TABLE XIX

Decrease in PFA Levels of Sub-Cellular Fractions of
Rat¹ Heart² and Brain³ Homogenates

Sub-Cellular fraction	Mg PFA per gm. of tissue		
	Before incuba- tion	After 180 min. incubation	Percent decrease during incubation
<u>Brain:</u>			
Mitochondria	1.0083(3) ⁴	0.9159(3)	8
Microsomes	0.4098(3)	0.2216(3)	45
<u>Heart:</u>			
Mitochondria	3.0914(3)	2.9286(3)	5
Microsomes	2.5892(3)	2.0871(3)	20

¹Rats used were three months old.

²Heart homogenate concentration = 25 mg/ml.

³Brain homogenate concentration = 50 mg/ml.

⁴Brackets indicate number of experiments.

This would tend to present a false picture as to the cellular location of in situ peroxidation. Although the tissue environment is somewhat different than the test solution (tris buffer) in which these sub-cellular particles are separated and suspended, it seems unlikely that this change in the environment would affect the results very much.

The conclusion that the microsomal-supernatant fraction is the site of maximum lipid peroxidation is supported by the observations that the fraction contains the highest level of TBA-positive material, both before and after incubation, and that this fraction also sustains the greater loss of PFA during incubation (Tables XVIII and XIX).

When the brain microsomes were spun down and the two fractions thus obtained were studied separately for the peroxidation products (Table XVIII), low TBA-positive values were obtained. The microsomes supplemented by a dialysate of the supernatant, produced amounts of TBA-positive material equivalent to those produced by the microsomal-supernatant fraction. However, the dialyzed supernatant did not have any effect on the production of TBA-positive material by the microsomes. Kitabchi et al. (16) reported that heating the supernatant from rat liver destroyed most of its ability to form TBA-positive material when incubated with microsomes, but that the addition of FeSO_4 to the heated supernatant restored its activity. This suggests the possibility that the essential factor(s) in the supernatant are metal ions.

In another series of experiments, the sub-cellular particles were separated after incubation of the whole rat brain and heart homogenate for 180 minutes (Tables XX and XXI).

There was no significant difference in the levels of TBA-positive material in these sub-cellular particles whether separated before or after incubation, with the exception of the supernatant-fraction. The TBA-positive material of the supernatant was much higher when separated after incubation. As already noted, the supernatant probably contains metallic ions which catalyze the lipid peroxidation in the microsomes and perhaps also in the mitochondria. During incubation, the TBA-positive material (MA) produced in the particulate fraction, is released into the supernatant, being water soluble. This would account for the high level of TBA-positive

TABLE XX

Distribution and Formation of Lipid Peroxidation Products from
Homogenates of Rat¹ Brain and Heart Sub-Cellular
Fractions Separated before and after Incubation

Sub-Cellular Fraction	Absorbance at 535 m μ per 100 mg. of tissue			
	Before incuba- tion B_0^a	Incubated for 180 minutes B_{180}^b	Separated after 180 min. incu- bation B_{inc}^c	" B_{inc} " further incubated for 180 minutes ^d
<u>Brain:</u>				
Mitochondria	0.078	0.068	0.050	0.050
Microsomes	0.046	0.046	0.040	0.040
Supernatant	0.100	0.122	0.670	0.690
<u>Heart:</u>				
Mitochondria	0.084	0.100	0.080	0.084
Microsomes	0.088	0.128	0.102	0.108
Supernatant	0.108	0.124	0.144	0.144

¹Normal rats used were about 4 months old.

^aThe sub-cellular fractions were separated immediately after sacrificing the rat.

^bFractions were separated as in (a) and were then incubated for 180 mins.

^cFractions were separated from the whole homogenates which had been incubated for 180 minutes.

^dFractions were separated as in (c) and were then incubated for an additional 180 minutes.

TABLE XXI

Decrease in PFA Levels of Brain and Heart Sub-Cellular Fractions
Separated before and after Incubation

Sub-Cellular fraction	Mg PFA per gm. of tissue			Percent Decrease between (b) and (c)
	Before incuba- tion	Incubated for 180 minutes	Separated after 180 min. incu- bation ^c	
	B ₀ ^a	B ₁₈₀ ^b		
<u>Brain:</u>				
Mitochondria	1.0304	1.0304	0.3963	60
Microsomes	0.4623	0.3305	0.0660	80
<u>Heart:</u>				
Mitochondria	3.0914	2.1927	2.1927	0
Microsomes	2.5892	2.0608	1.8494	10

a,b,c: Same as described in Table XX.

material in the supernatant after incubation of the whole homogenate.

Most of the TBA-positive material probably comes from the microsomal fraction, with less if any, coming from the mitochondria. This point is substantiated by the marked percent decrease in PFA level of the microsomes produced by incubation (Table XXI). The percent decrease is higher in the microsomal than in the mitochondrial fraction.

Heart tissue from the normal rat does not exhibit the marked degree of peroxidation as does brain tissue (Tables XX and XXI). More about this differentiation will be discussed in Sections B and C.

4. General Discussion

The TBA test has been used by different investigators (6,8,124) in many different ways, which makes the comparison of results from different laboratories difficult. For example, preliminary studies during the present investigation showed that the buffer system used (Tris, phosphate, KCl) can have a significant effect on the estimation of tissue TBA-positive material, and it was for this reason that a single buffer (Tris) was employed for all the work described here.

The manner in which tissue was handled prior to the application of the TBA test was considered to be of importance. It was noted that different investigators have handled tissue in widely divergent ways in studies of this nature. It was felt that in comparative studies using this test, it was advisable to use a fixed homogenate concentration. Although the TBA test is not quantitative, it is felt that it provides a reasonably good estimate of peroxidation, provided the conditions with respect to both the tissue pretreatment and the test itself are rigorously controlled.

Studies of the autoxidation of pure UFA indicate that the process is initiated by oxygen, and is perpetuated through free radical mechanisms (18,75). The oxidation is accelerated by certain metal ions, of which iron appears to be one of the most effective (18,75). The overall process in living tissue is probably much more complex, although it is quite likely that the basic chemistry is similar. The regulation of O_2 tension, metallic ion balance, presence or absence of antioxidants, or reactions producing suitable redox environments could all influence the in vivo formation of

peroxides and their decomposition products. The present study has confirmed previous observations concerning the catalytic effect of iron and ascorbate ions. It has also shown that vanadyl ions have an accelerating effect on lipid peroxidation reactions, and that although ferric ions were more efficient, both ferrous and ferric ions accelerated the process.

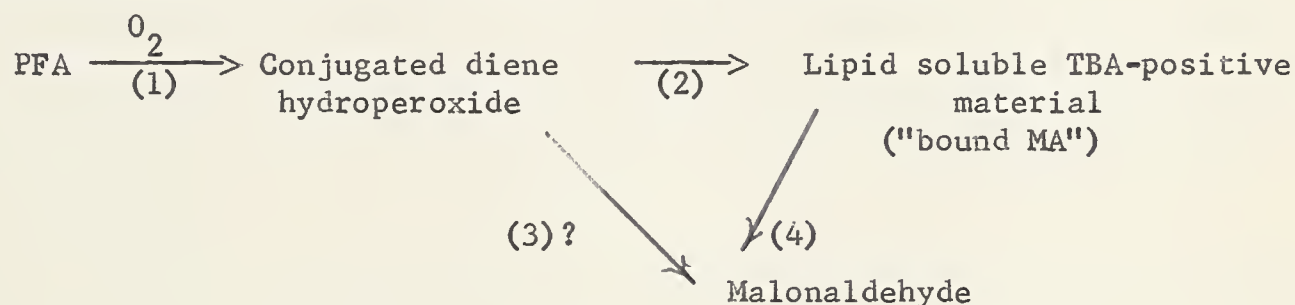
Heavy metals in brain are probably associated with complex molecules. Tappel and his associates (6,75,123) have indicated that a hematin catalyzed oxidation of lipid occurs in liver preparations from both rat and rabbit. The cytochromes, themselves vulnerable to autoxidation (126) could act as catalysts in brain lipid oxidations.

Ottolenghi (8) suggests that the mechanism in rat liver mitochondria is non-enzymatic and involves co-oxidation of ascorbic acid and unsaturated lipid mediated by a metal ion, probably iron. Considerable work by another group (16,108,127,128) has demonstrated conclusively that ascorbic acid synthesis in rat liver preparations is depressed in conditions where lipid peroxides are present. They also found (127) that metal ions (cobalt, manganese, iron) greatly influenced the levels of both ascorbic acid and peroxidation products in their preparations. Ascorbic acid formation takes place in the microsomal fraction of the rat liver cell (129). The present study has shown that the microsomal-supernatant fraction exhibits high levels of lipid peroxidation breakdown products (Table XVI). It has been suggested that the intermediary products of ascorbate synthesis (L-gulonolactone and L-xylohexulonolactone) may be more closely associated with cellular lipid reactivity than is ascorbic acid itself (128). The addition of ascorbic acid to brain homogenates does increase the amount of peroxidation products in that tissue (see Tables XV and XVI). Adult rat brain cortex contains relatively large amounts of ascorbic acid (130) but it is not known whether or not brain can synthesize ascorbic acid at any stage of growth.

Boiling tissue homogenates for 10 minutes at 100°C caused a rapid development of TBA-positive material. However, these heated homogenates, on incubation at 37°C, produced additional TBA-positive material. This strongly suggests that in vitro lipid peroxidation is a metal catalyzed, non-enzymatic process.

The absorption spectra of the TBA-chromogen formed from brain and heart homogenates, their lipid extracts, and pure linolenic acid, were similar but different from the TBA-chromogen formed from pure malonaldehyde. The former have an absorption maxima at 450 mμ in addition to the one at 535 mμ, which is due to the MA-TBA chromogen. This indicates that these tissues may contain some other TBA-positive material besides malonaldehyde. The identity of this material remains unknown. There is a possibility that this TBA-positive material might be a precursor(s) of malonaldehyde. Recently Taufel and Zimmermann (131), studying the reaction of various carbonyl compounds with TBA, found that heptanal, 2-heptenal and 2,4-heptdial yielded chromogens with absorption maxima at 452 mμ.

The results of aerobic incubation of brain and heart tissue homogenates and their lipid extracts (Table XIII) suggest that the following reaction sequence could be operative during incubation:



During incubation there is only a very small decrease in PFA concentration (reaction (1)) but a large increase in free malonaldehyde. Lipid extracts of whole homogenates contained high levels of lipid soluble TBA-positive material (bound malonaldehyde). After 2 hours' incubation, these extracts contained about the same amounts of this material but the level of conjugated diene hydroperoxide had decreased considerably. Incubation of cold TCA-extracts of tissue homogenates produced no increase in the amount of TBA-positive material contained therein. Cold TCA-extracts of incubated (120 min., 37°C) homogenates contained much higher levels of TBA-positive material than did extracts of unincubated homogenates. These observations are compatible with the premise that during incubation reaction (4) is operative, causing an increase in free, water-soluble malonaldehyde, while

at the same time, reaction (2) functions to keep the level of lipid-soluble TBA-positive material relatively constant. It may be that the primary effect of metals such as iron and vanadium is to accelerate reaction (4), i.e. the release of bound malonaldehyde, rather than to increase the rate of reaction (1). The studies described here indicate that the initial effect of the addition of iron salts to the tissue homogenates is to increase the level of TBA-positive material markedly, while leaving unaltered the concentration of PFA.

The results, therefore, strongly suggest that the events occurring during aerobic incubation are concerned primarily with the breakdown of preformed peroxidic compounds, rather than with further PFA destruction.

Section B

Influence of Vitamin E Deficiency on Lipid Peroxidation in Rat Tissues

Cole in 1956 (3) and Kibrick in 1959 (4) reported the presence of the products of lipid peroxidation in embryonic and adult tissues of Golden hamsters. Dam and Granados in 1945 (1) had noted that the unsaturated lipids tended to peroxidize in tissues of rats on vitamin E deficient diets. Bernhard in 1958 (2) claimed that vitamin E had no effect on the PFA levels in either brain or liver. The present study has, at least in part, shown that vitamin E deficiency does cause both a decrease in tissue PFA and an increase in the susceptibility of tissue lipids to peroxidation in most rat tissues.

1. General tissue survey

The PFA level decreased in tissues from rats maintained on a vitamin E deficient diet, with the exception of brain, in which no change was observed (Table XXII). The TBA-positive material, on the other hand, was in general increased in tissues from vitamin E deficient animals. There was, however, great variability among the various tissues, with heart and adrenals showing the greatest and spleen and thymus little, if any, change (Table XXIII).

Discussion:

Brain: The brain tissue exhibited a very high level of TBA-positive material in vivo and great increase in vitro, that is, after aerobic incubation. But there was no difference between the values obtained with brains removed from control and vitamin E deficient rats. Bieri (25) reported somewhat similar findings. The PFA levels in the brains of rats maintained on the two diets did not differ. An adult rat brain contains relatively large amounts of PFA - of the order of 9-10 mg. per gm. of brain, and weighs about one gm.

Heart: Very low levels of TBA-positive material were found in freshly excised heart tissue, and only a slight increase resulted from the in vitro incubation of tissue from normal rats. Incubation of homogenates of heart tissue from vitamin E deficient rats, on the other hand, produced much greater increases in the level of TBA-positive material. Less PFA was found in hearts from vitamin E deficient rats than in those of control

TABLE XXII

PFA Levels in Tissues from Control and Vitamin E
Deficient Rats¹

Tissue	Type Control (C) Deficient (D)	Before incubation Mg PFA per gm. of tissue	Wet weight of tissue in gms.
Brain	C	9.1900 (6) ²	1.1000
	D	9.0090 (6)	1.0090
Heart	C	10.1188 (6)	0.550
	D	8.0050 (6)	0.350
Liver	C	6.9400 (6)	-
	D	5.7500 (6)	-
Kidney	C	8.3940 (3)	2.1520
	D	5.1558 (3)	1.0215
Thymus	C	5.3930 (3)	0.3620
	D	3.3840 (3)	0.0935
Lung	C	1.8900 (3)	0.6265
	D	1.6260 (3)	0.3945
Spleen	C	1.7970 (3)	0.5725
	D	1.0840 (3)	0.3615
Adrenal	C	16.40 (3)	-
	D	4.10 (3)	-

¹Rats used were 9 weeks old and had been on diet for 6 weeks.

²Brackets indicate number of experiments.

TABLE XXIII

Lipid Peroxidation in Normal and Vitamin E Deficient Rat¹
Tissues as Estimated by TBA Test

Tissue	Type Control (C) Deficient (D)	Absorbance at 535 m μ per 100 mg. of tissue	
		Before incubation 0 time	After incubation 120 mins.
Brain	C	0.188 (6) ²	0.680 (6)
	D	0.196 (6)	0.726 (6)
Heart	C	0.108 (6)	0.156 (6)
	D	0.124 (6)	0.900 (6)
Liver	C	0.084 (6)	0.136 (6)
	D	0.084 (6)	0.154 (6)
Kidney	C	0.172 (3)	0.218 (3)
	D	0.200 (3)	0.236 (3)
Thymus	C	0.124 (3)	0.534 (3)
	D	0.134 (3)	0.406 (3)
Lung	C	0.100 (3)	0.126 (3)
	D	0.120 (3)	0.284 (3)
Spleen	C	0.350 (3)	0.390 (3)
	D	0.208 (3)	0.246 (3)
Adrenals	C	0.500 (3)	0.600 (3)
	D	0.520 (3)	15.000 (3)

¹Rats used were 9 weeks old and had been on diet for 6 weeks.

²Brackets indicate number of experiments.

animals. Hearts from control and vitamin E deficient rats weigh about 0.5 and 0.3 gm. respectively.

Other tissues: Of the other tissues studied, all showed the presence of some amount of peroxidation products in vivo. Adrenals from vitamin E deficient animals were most susceptible to peroxidation in vitro.

Polyunsaturated fatty acids are the main substrate of the lipid peroxidation reaction (3,22). When the TBA-positive content is related to the tissue PFA level, definite differences between control and deficient tissues were noted in vivo, with the exception of the brain (Table XXIV).

The polyunsaturated fatty acids of the heart and adrenals seem to be particularly susceptible to peroxidation. It appears, therefore, that while most tissues from vitamin E deficient rats have a decreased PFA content, the remaining PFA exhibits an increased susceptibility to peroxide formation. This loss of PFA in the tissues from vitamin E deficient rats might be accounted for, in part, by an increase in peroxidation products, which in turn are liberated (converted to TBA-positive material) during incubation. This may well be a result of a decrease in the level of a natural tissue antioxidant, i.e. vitamin E.

Conclusion: All normal rat tissues tested exhibited some evidence of lipid peroxidation in vivo, and most of them showed a susceptibility to peroxidation in vitro. However, susceptibility of tissue lipids to peroxidation was much greater in tissues from rats kept on vitamin E deficient diet. Brain is an exception. In this tissue, neither the level nor the vulnerability to peroxidation of the PFA appeared to be affected by the diet on which the experimental animal was maintained.

2. Influence of vitamin E deficiency on lipid peroxidation in sub-cellular fractions of the rat brain and heart

It has already been shown that the most active sub-cellular fraction in promoting lipid peroxidation in normal rat brain and heart is the microsomal-supernatant fraction (see Section A). Studies on the sub-cellular fractions from tissues of vitamin E deficient and normal rats were carried out, and the results are summarized in Table XXV.

TABLE XXIV

Lipid Peroxidation in Normal and Vitamin E
Deficient Rat Tissues

Tissue	Type Control (C) Deficient (D)	Absorbance at 535 m μ per mg. PFA	
		Before incubation	After 120 min. incubation
Brain	C	0.206	0.747
	D	0.217	0.805
Heart	C	0.106	0.154
	D	0.155	1.125
Liver	C	0.121	0.196
	D	0.146	0.268
Kidney	C	0.205	0.259
	D	0.388	0.458
Adrenal	C	0.304	0.365
	D	1.268	36.58

TABLE XXV

Influence of Vitamin E Deficiency on Lipid Peroxidation in
Sub-Cellular Fractions of the Rat¹ Brain and Heart

Sub-cellular fraction	Absorbance ² at 535 mμ per 100 mg. of tissue			
	Control		Deficient	
	0 time	180 min.	0 time	180 min.
<u>Brain:</u>				
Mitochondria	0.068	0.076	0.064	0.076
Microsomes	0.052	0.058	0.056	0.066
Supernatant	0.110	0.120	0.120	0.120
Supernatant- microsomes	0.136	0.444	0.174	0.450
<u>Heart:</u>				
Mitochondria	0.088	0.100	0.076	0.108
Microsomes	0.084	0.112	0.080	0.100
Supernatant	0.108	0.132	0.116	0.216
Microsomes- supernatant	0.100	0.120	0.128	0.384
	Mg PFA per gm. of tissue			
<u>Brain:</u>				
Mitochondria	1.0083	0.9159	1.0104	0.9008
Microsomes	0.4098	0.2216	0.4195	0.2300
<u>Heart:</u>				
Mitochondria	3.0914	2.9298	-	-
Microsomes	2.5892	2.0871	2.2192	1.1360

¹Rats on diets for 12 weeks.

²Results are average of 3 sets of experiments.

The brain microsomal-supernatant fraction exhibited high level of TBA-positive material in vivo, which increased markedly after incubation, but there was no significant difference between the values obtained with preparations from normal and deficient brains. The heart, on the other hand, showed much less evidence of in vivo peroxidation in both vitamin E deficient and the control rats. After aerobic incubation, however, the level of TBA-positive material was greatly increased in the microsomal-supernatant fraction from the vitamin E deficient rat heart, while the increase in the corresponding fraction from normal rat heart was small.

The PFA levels, both in heart and brain sub-cellular fractions decreased with corresponding increase in TBA-positive material. This behavior is similar to that observed with the whole homogenates.

Discussion: It has been shown that in homogenates of heart tissue from rats deficient in vitamin E, maximum production of TBA-positive material takes place in the microsomal-supernatant fraction. It is quite possible that in vivo, some lipid peroxidation takes place in other cellular fractions (mitochondria for example), with the release of TBA-positive material into the supernatant. However, this should not detract from the significance of the observation that in vitro, both production of TBA-positive material and loss of PFA is much greater in the microsomal-supernatant than in the mitochondrial fraction.

3. Effect on lipid peroxidation in rats fed on corn-oil supplemented diet

The results of experiments designed to examine the effect of dietary corn oil on lipid peroxidation in rat tissues are summarized in Tables XXVI and XXVII. Four groups of rats were maintained on the following diets:

- (i) Normal diet
- (ii) Normal diet + corn oil
- (iii) Vitamin E deficient diet
- (iv) Vitamin E deficient diet + corn oil

The addition of corn oil to the deficient diet did not restore PFA levels in either heart or plasma to normal. However, addition of corn oil to the control diet produced higher than normal tissue PFA content, as one might have expected.

TABLE XXVI

PFA Content of Tissues from Rats¹ Fed Corn Oil Supplemented Diets²

Diet	PFA as percent of control ³			Rat weight ⁴ (gms.)
	Brain	Heart	Plasma	
Control	100	100	100	137
Control + oil	103	110	146	137
Deficient	100	40	36	93
Deficient + oil	101	60	62	88

¹Values are mean of 4 different sets of experiments.

²Corn oil added to diets at 4% w/w.

³Rats were killed when they were 6 weeks old and had been on the diet for 3 weeks.

⁴The weight of rat varied with the type of diet it was fed on.

TABLE XXVII

Lipid Peroxidation in Tissues from Rats¹ Fed Corn Oil²
Supplemented Diets

Diet	Absorbance at 535 mμ per 100 mg. of tissue			
	Brain		Heart	
	0 time	180 min.	0 time	180 min.
Control	0.200	0.738	0.088	0.154
Control + oil	0.206	0.720	0.096	0.344
Deficient	0.206	0.816	0.104	0.640
Deficient + oil	0.208	0.792	0.120	0.552

¹Rats were killed when they were 6 weeks old and had been on the diet for 3 weeks. Values are mean of 4 different sets of experiments.

²Corn oil added to diets at 4% w/w.

Discussion: In chicks, it has been shown that the onset of vitamin E deficiency can be hastened by the addition of unsaturated fatty acids to the diet (132,133,134). The level of peroxidation products in heart tissue from rats maintained on the corn-oil supplemented vitamin E deficient diet was only slightly higher than that in heart tissue from rats on the vitamin E deficient group (Table XXVII). In view of the increased intake of PFA by rats fed the corn-oil supplemented diet, a difference larger than the one observed was anticipated. An explanation for the unexpected result may lie in the finding (made after the experiment was terminated) that the corn oil used contained a small amount of tocopherol. Nevertheless, it is clear that the level of PFA in the heart tissue of rats maintained on a vitamin E deficient diet cannot be restored to normal levels by the addition of PFA to the diet. Neither the PFA levels nor the formation of TBA-positive material in brain appear to be affected by dietary changes.

Conclusion: It appears that a lack of vitamin E leads to the loss of PFA from most tissues presumably by lipid peroxidation. Brain tissue is the outstanding exception to this rule. Furthermore, dietary PFA cannot restore tissue PFA levels to normal in the vitamin E deficient rats.

4. General discussion

The experimental work reported here has been concerned with changes in the lipids of vitamin E deficient rat tissues prior to the onset of overt and obvious deficiency symptoms. This is the period when the rats have been maintained on a vitamin E deficient diet for 6-10 weeks.

The chief substrates for peroxidative attack are the PFA, which must be obtained from the diet or formed from essential fatty acids in vivo. Vitamin E deficiency resulted in decreased amounts of polyunsaturated fatty acids in most of the tissues examined. The data suggest that this decrease is due to an accelerated rate of in vivo peroxidation in the deficient animals. Increasing the dietary PFA did not restore PFA levels to normal in the heart and plasma of vitamin E deficient rats. In chicks, such feeding has been reported to accelerate the onset of deficiency symptoms (132,133,134). These can be alleviated only by feeding adequate

amounts of antioxidants or antioxidant forming materials (Bieri and Anderson, (132,134)). The PFA have been implicated as structural units in the cell particulate systems. If this is so, it would be expected that such tissues would tend to function abnormally in the face of a vitamin E deficiency, and that eventually this dysfunction would result in the obvious damage found in terminal vitamin E deficiency.

Though many antioxidants of diverse structure can lessen the severity of vitamin E deprivation symptoms, none can completely replace the vitamin (135). This had led some workers to postulate that the vitamin has functions other than that of an antioxidant (Nason, 109, 110, 114). However, these theories are largely unsupported by experimental evidence and recent studies strongly suggest that all the aberrations associated with vitamin E deficiency can be explained on the basis of a lack of proper tissue anti-oxygenic capacity (Tappel, 136).

These studies, as well as others (124,136) have demonstrated that brain contains large amounts of lipid peroxidation products. However, the concentration of this material is not affected by either vitamin E deficiency (present work) or vitamin E feeding. Bieri has suggested that adult chick brain is impermeable to tocopherol (25). This could account for the high level of TBA-positive material in this tissue. However, chick brain is highly susceptible to encephalomalacia caused by diets high in PFA, indicating that the substrate for peroxidation reaches the brain readily. The situation in the rat seems to be different. The present work (Section C) showed that the level of peroxidation products reaches a maximum during the early stages of brain development. The leveling off in the level of TBA-positive material may well be related to the formation of the blood-brain-barrier (if there is any) which would tend to limit the entry of both accelerators and inhibitors of peroxidation to the brain. The result, whatever the cause, is that the peroxidation of brain lipids in the adult rat are little influenced by dietary vitamin E.

Adrenals and heart from vitamin E deficient rats were found to be exceptionally susceptible to lipid peroxidation in vitro.

Section C

Examination of Rat Brain and Heart During Early Growth

1. Lipid stability in brain and heart

The relatively high level of TBA-positive material and the large quantity of PFA - the major substrate for the peroxidation reaction - in rat brain, stimulated an investigation into the production and concentration of the TBA-positive material in the maturing rat brain.

a. Brain: The concentration of PFA in the brain was found to increase approximately fourfold during the first 26 days of life (Figure 9). This increase in concentration is not as marked as is the increase in total brain PFA (Figure 10). The latter values are based on brain weight, the increase of which follows a sigmoidal pattern (Figure 10). Growth rate is greatest during this period in which active myelination is taking place in the white matter of the cerebral hemisphere.

The amount of TBA-positive material present per unit weight of PFA by lipid peroxidation decreased with age (Figure 11). The liberation of TBA chromogen during incubation is shown in the upper curve of Figure 11. This production, or release, is much reduced in older rats.

Figure 12 is constructed from data already illustrated in Figures 10 and 11, and shows the relationship between the age of the rat and (a) the amount of TBA-positive material present in freshly excised brain, and (b) the amount of TBA-positive material produced during incubation of the brain homogenate for 120 minutes.

It would appear that the amount of TBA-positive material, that is, the lipid breakdown products in the cerebral tissue increases until about the 10th day postpartum, after which it remains virtually constant although the weight and size of the brain continues to grow (see Figure 10). The amount in TBA-positive material produced during aerobic incubation of brain homogenate increases with increasing age of the animal in a manner similar to that observed in the case of endogenous TBA-positive material (Figure 12, 120 minute values). The maximum level in this case, is reached about the 15th day postpartum. It appears, therefore, that peroxidation events in rat cerebral hemispheres, remain relatively constant after the 10th to 15th day of age. Cole (3), studying peroxidation in

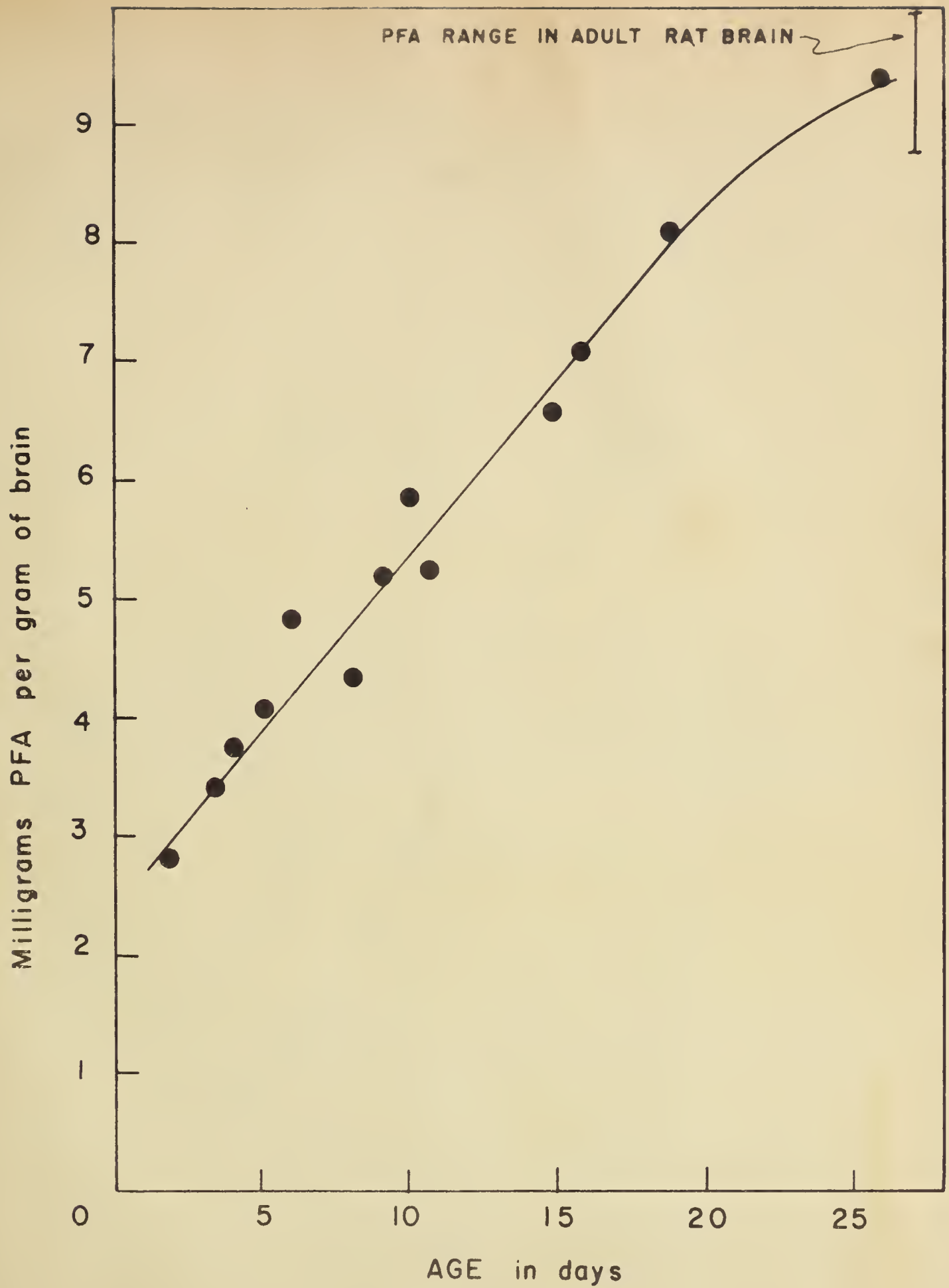


Figure 9

Increase in the Concentration of Polyunsaturated Fatty
Acids with Growth of Rat Brain

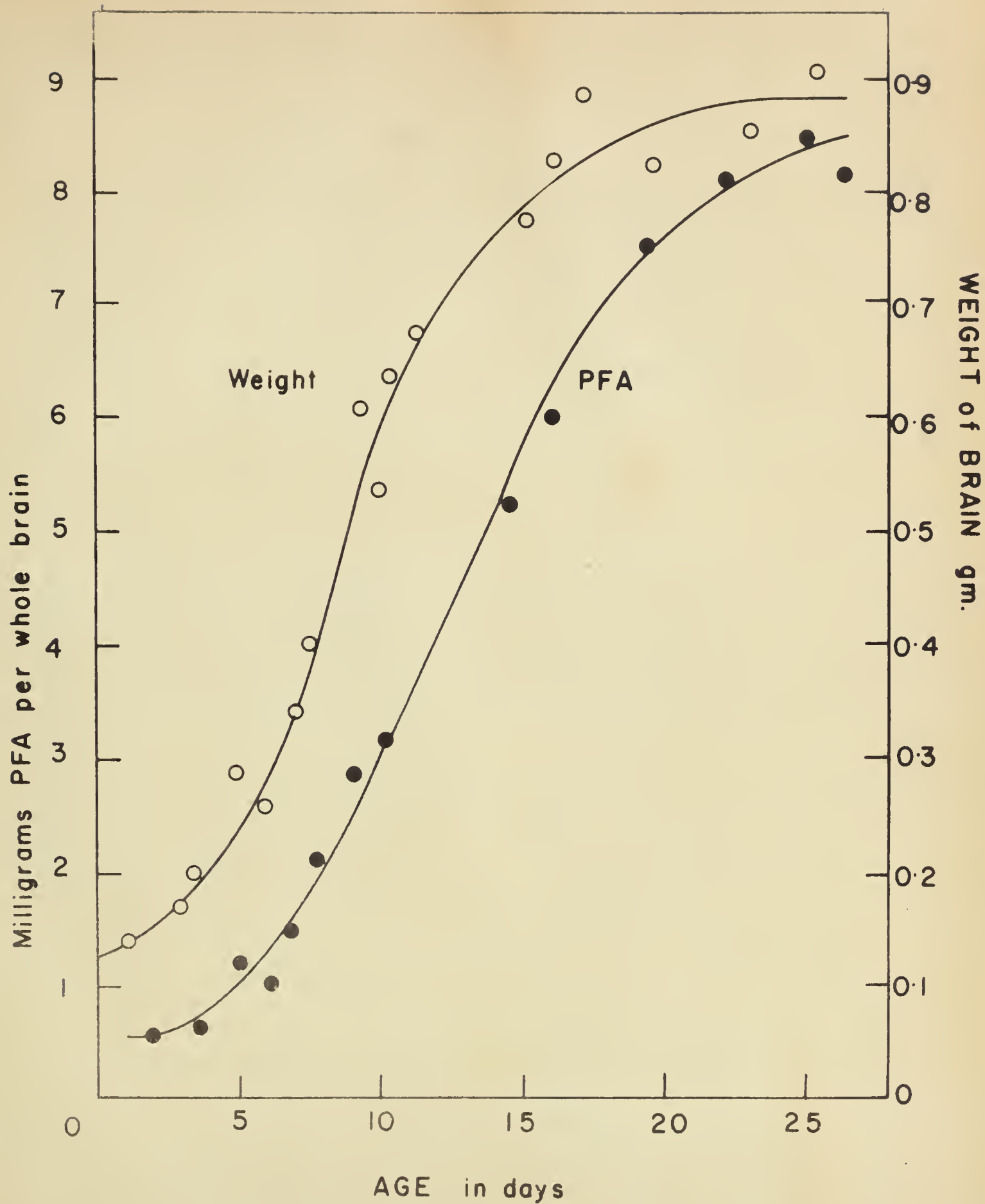


Figure 10

The Total Amount of PFA in Whole Rat Brain at Various Stages of Growth and Increase in Wet Weight with Age of the Same Tissue

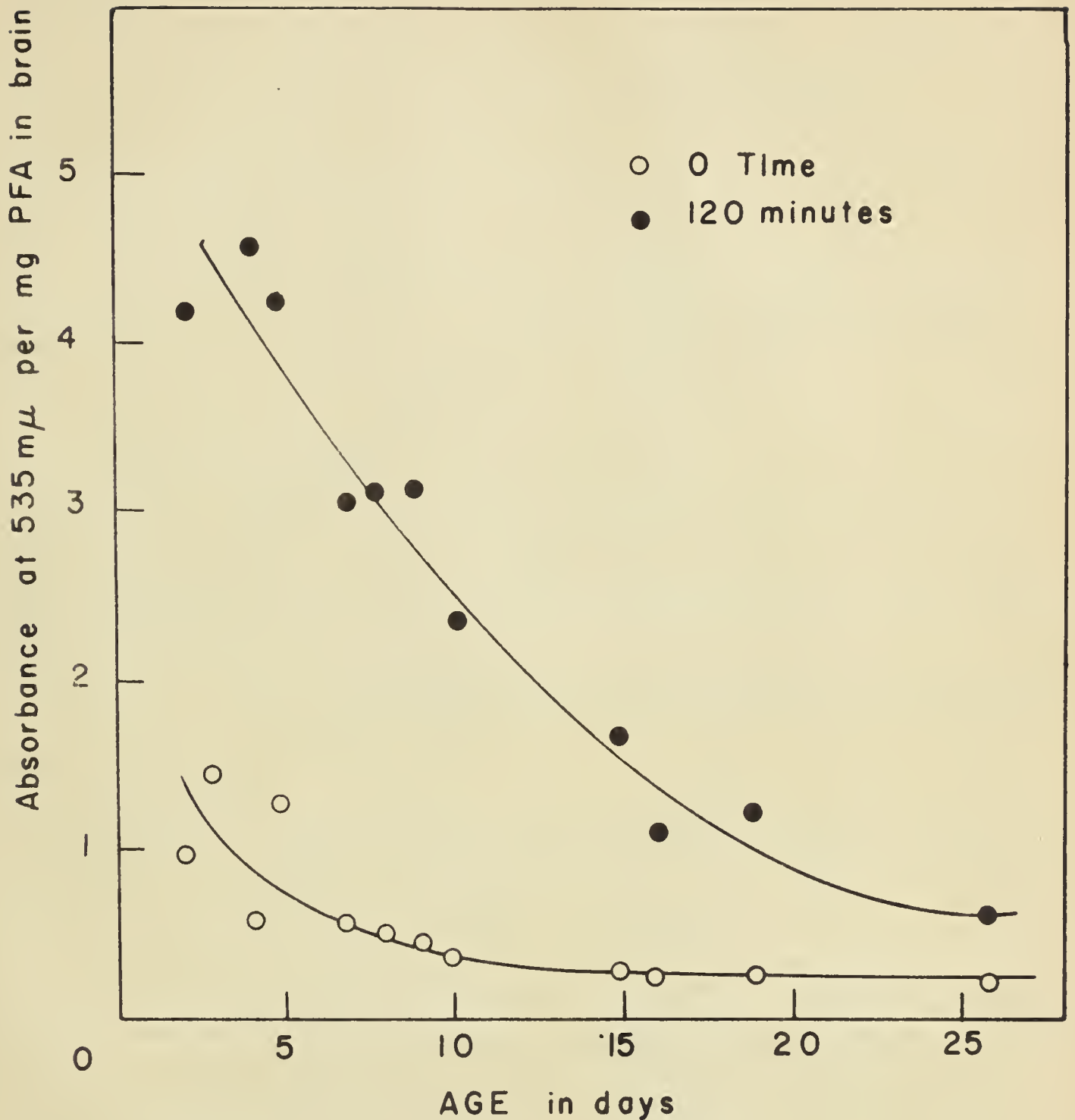


Figure 11

The Amount of TBA-Positive Material (Peroxidation Product) Per Unit Weight of PFA in Rat Brain at Various Stages of Growth. (Open circles represent the values for endogenous material. Closed circles represent the values after incubation.)

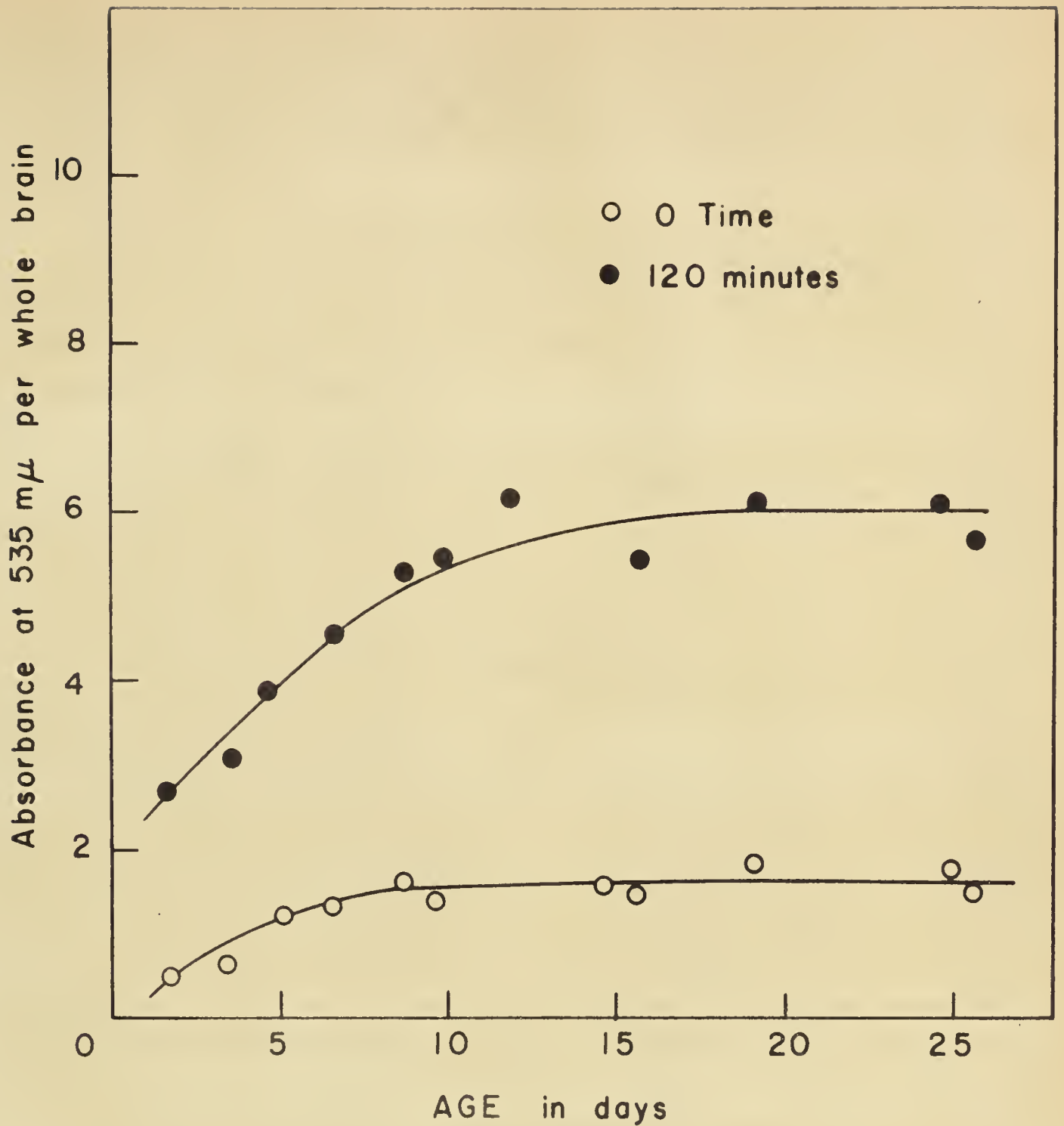


Figure 12

The Total Amount of TBA-Positive Material (Peroxidation Product)
in Whole Rat Brain at Various Stages of Growth.
(Open circles represent the values for endogenous material.
Closed circles represent the values after incubation.)

Golden hamster brain, observed that peroxide concentration in the brain of these animals reached a maximum about 10 to 16 days after birth. This is in reasonable agreement with the data arising from the present study with rat brain.

The results indicate that although the concentration of PFA in rat brain increases during the first 26 days of life, their tendency to peroxidize steadily decreases during this period. The net result is that the in situ level of lipid peroxidation products remains constant after the rats are 10 days old.

b. Heart: The concentration of PFA in the heart increased steadily with age (Figure 13). The level of PFA per whole heart increases most markedly from the 10th to 25th day (Figure 14). In contrast to brain, in which the maximum PFA level and the maximum brain weight is reached by the 20th day to 25th day, heart continues to increase both in weight and in PFA level. By about the 25th day postpartum, the heart has acquired about half the total PFA and a little more than one-third of the total wet weight of a fully matured rat heart.

The amount of TBA-positive material produced per unit weight of PFA by lipid peroxidation decreased with increasing age of the rat (Figure 15). The formation of TBA-positive material during aerobic incubation is shown in the upper curve of Figure 15. This production is reduced in older rats.

Figure 16 was composed from the values in Figures 14 and 15. The accumulation of TBA-positive material in the heart tissue seems to continue until about the 20-25th day postpartum (as compared to the 10th day in the brain).

Figure 15 also shows the level of TBA-positive material in a fully matured rat heart, indicating therefore that although the concentration of PFA in rat heart increases even after the 25th day (Figure 14), their tendency to peroxidize decreases progressively up to the 20th day postpartum, after which the heart contains a constant level of TBA-positive material.

Discussion: It seems evident, therefore, that although brain and heart show different patterns of development in the growing rat, they

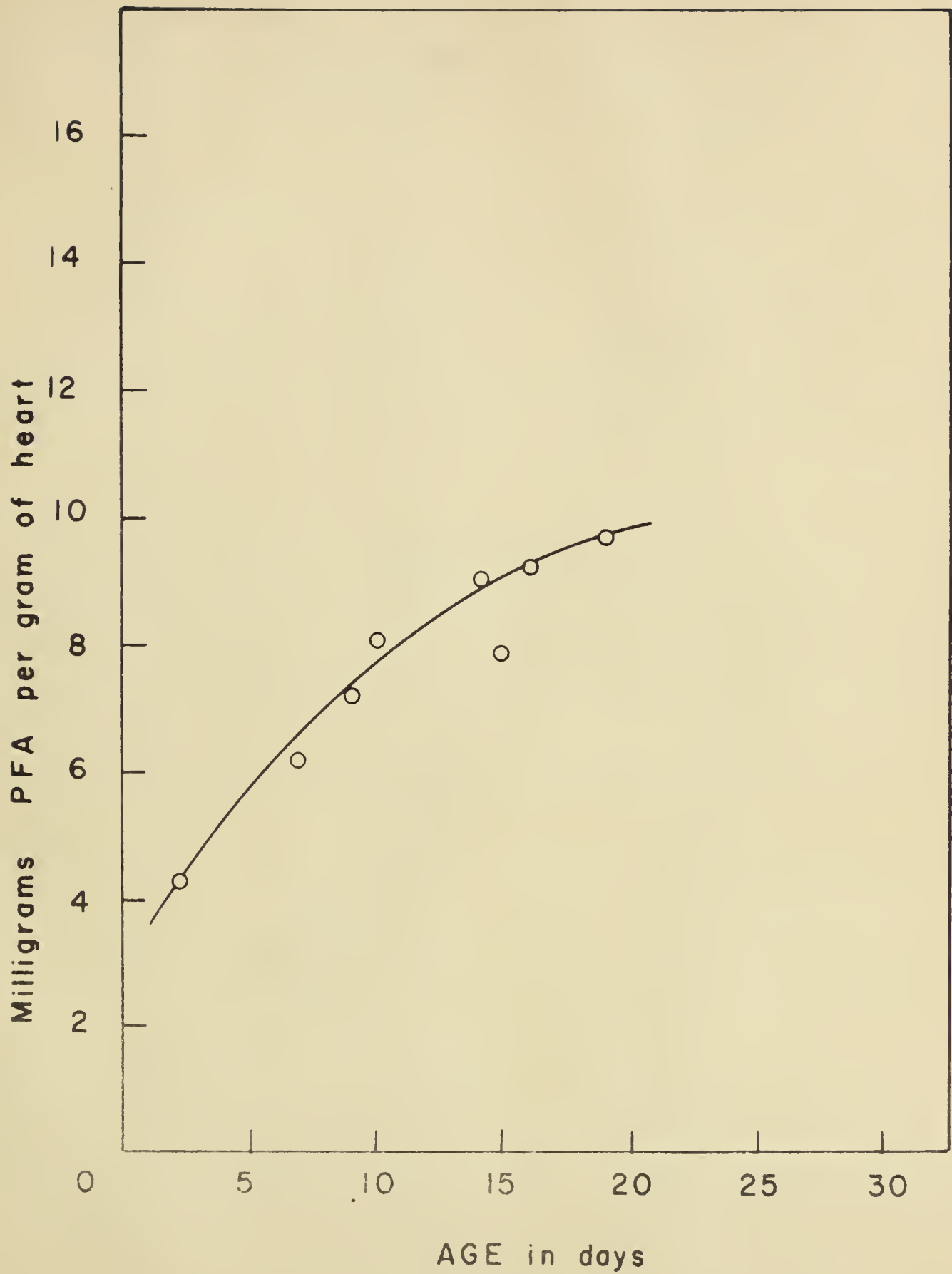


Figure 13

Increase in the Concentration of PFA with Growth of Rat Heart

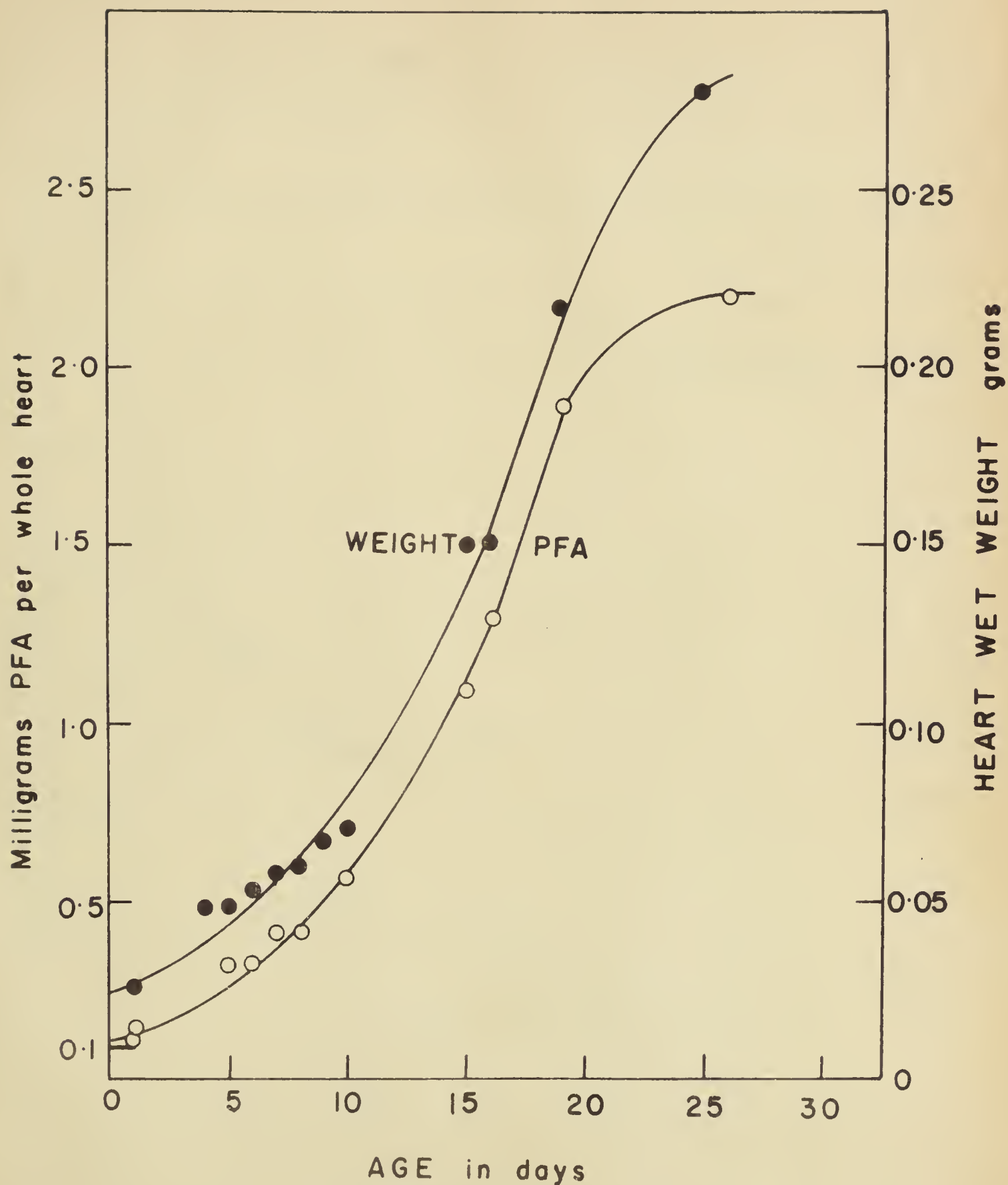


Figure 14

The Total Amount of PFA in Whole Rat Heart at Various Stages of Early Growth and Increase in Wet Weight of This Tissue with Age

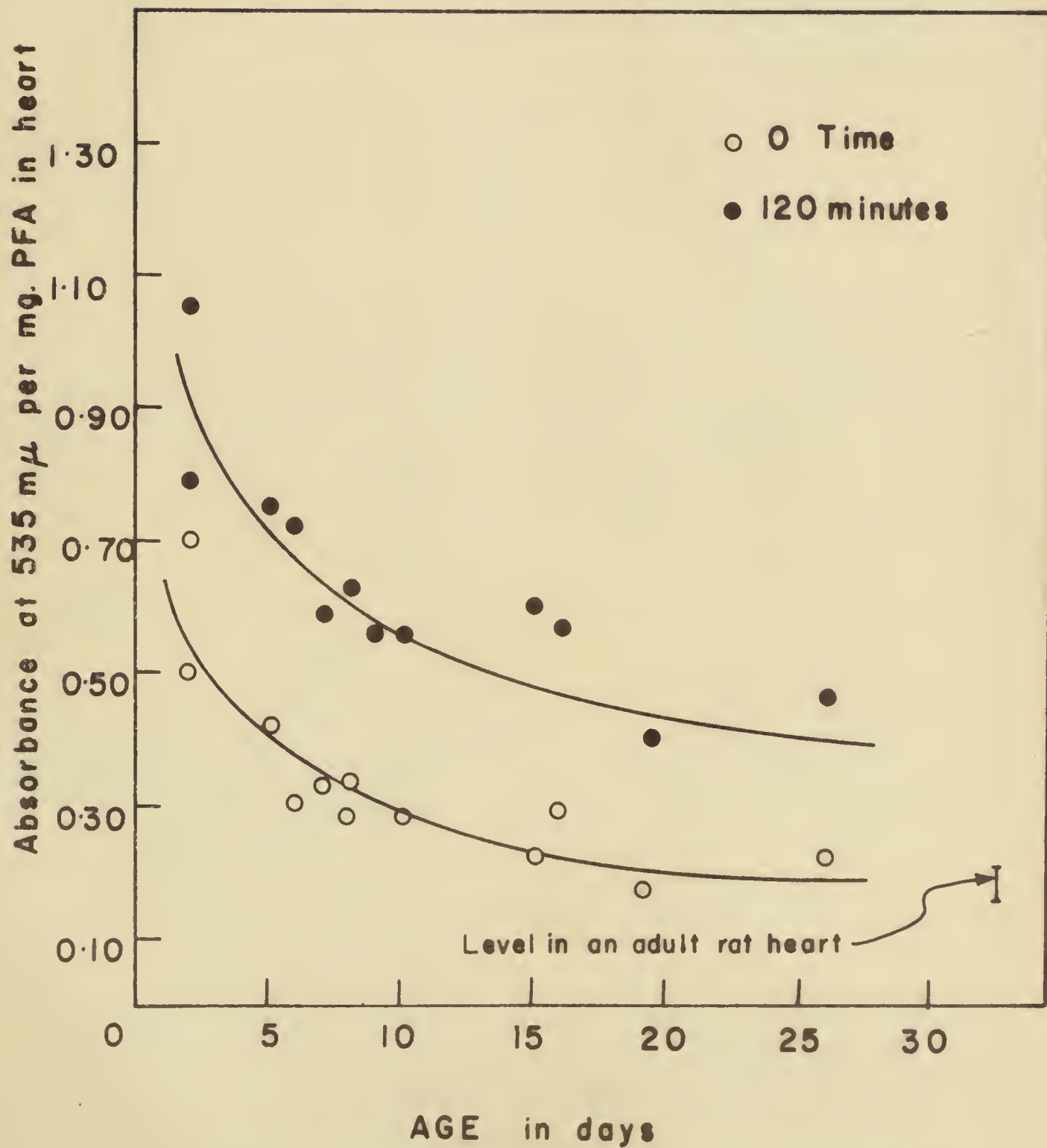


Figure 15

The amount of TBA-positive material (peroxidation product) per unit weight of PFA in rat heart at various stages of growth. Open circles represent the values for endogenous material. Closed circles represent the values after incubation.

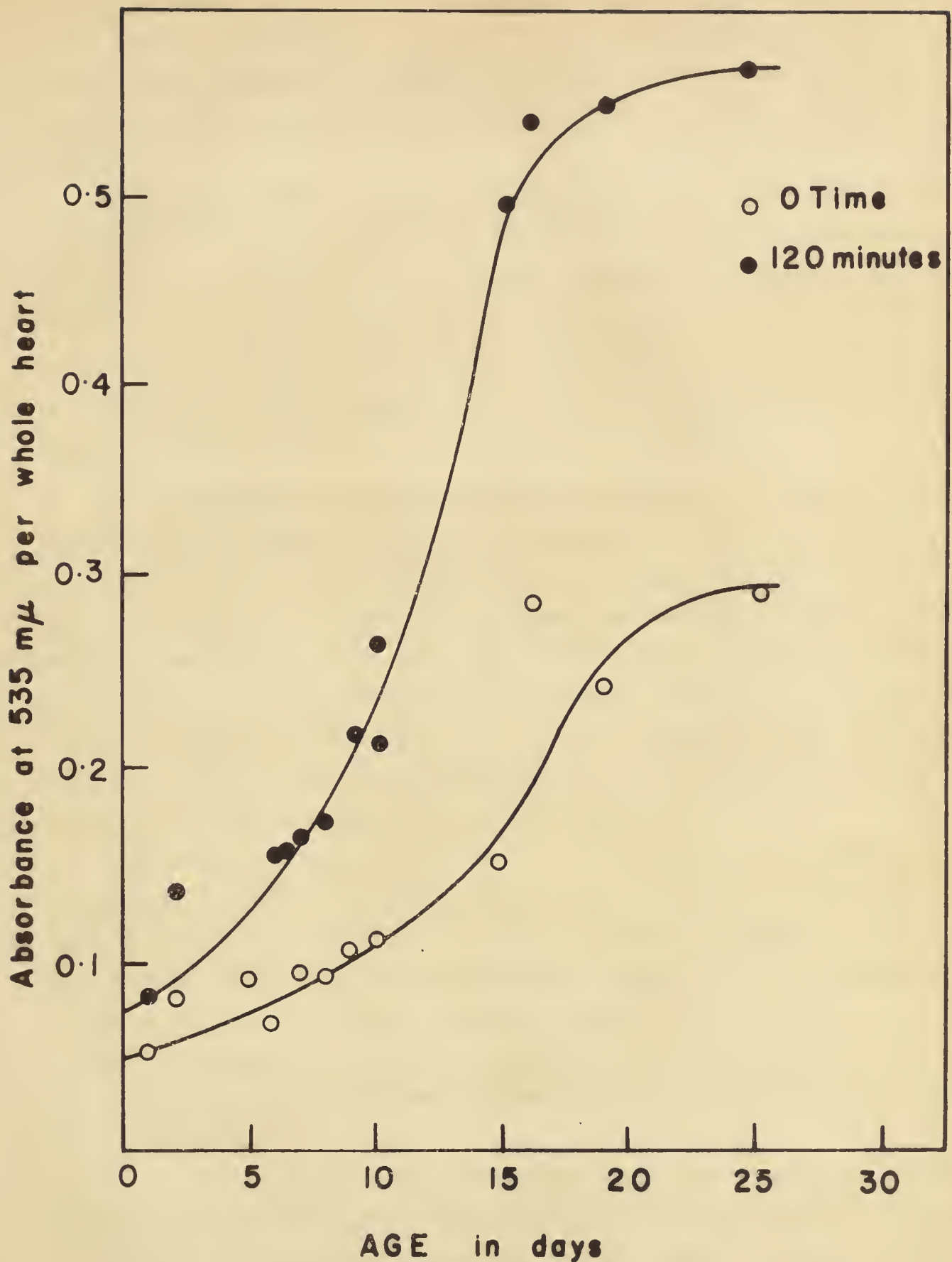


Figure 16

The Total Amount of TBA-Positive Material in Whole Rat Heart at Various Stages of Growth

(Open circles represent the values for endogenous material. Closed circles represent the values after incubation.)

exhibit similar trends insofar as the peroxidation of their polyunsaturated fatty acids is concerned. In both tissues, the tendency of PFA to undergo peroxidation decreases progressively during early growth, reaching stable, minimum levels at about the 10th and 20th days in brain and heart respectively.

Conclusion: The experimental results indicate that the production of TBA-positive material, apparently derived from the PFA peroxidation, decreases with maturation of rat brain and heart. It appears that during maturation, some factor or process is gradually introduced or generated within these tissues, which retards the tendency of unsaturated fatty acids to undergo oxidation in situ. The process is possibly related to the maintenance of PFA stability in adult rat brain and heart.

2. Studies of the levels of PFA and TBA-positive material in the microsomal-supernatant fraction of developing rat heart and brain

The results of these investigations are presented in Table XXVIII. Only the microsomal-supernatant fraction was studied, because previous studies (see Section B.2) indicated that it was the site of maximal lipid peroxidation. In the microsomal-supernatant fraction of both heart and brain, the pattern with respect to the relationship between the age of the animal and the levels of PFA and TBA-positive material, was similar to that observed with the corresponding whole homogenate.

Discussion: Tappel et al. (6,7) have reported that the mitochondrial fraction is the site of lipid peroxidation. Studies in this laboratory have demonstrated that the microsomal-supernatant fraction isolated from unincubated tissue homogenates possesses a high level of peroxidation products and that this level increases markedly on incubation. It was of interest, therefore, to examine the PFA levels and the peroxidation products in the microsomal-supernatant fraction of the developing rat brain and heart.

It is quite evident from the results that the tendency of the PFA of the microsomal-supernatant fraction of both brain and heart to peroxidize decreases with increasing age of the animal. This is in good agreement with the results of similar studies on the corresponding whole homogenates.

TABLE XXVIII

Relationship Between Peroxidation Products and PFA Levels in the
Microsomal-Supernatant Fraction of Rat Tissues,¹ and the
Age of the Animal

Age in days	Heart		Brain			
	Absorbance at 535 mμ per mg PFA in heart ²		mg PFA per gm. of heart		Absorbance at 535 mμ per mg PFA in brain ²	
	0 time	120 min.	0 time	0 time	120 min.	mg PFA per gm. of brain
2	2.080	4.288				
5	1.400	2.030				
6	1.288	2.900	1.1600	6.420	15.982	
8	1.034	2.178	2.1664			0.4755
10			2.1136	6.300	14.740	0.4887
11				5.450	7.752	
15	1.080	2.200	2.2193			
16			2.2457	2.482	7.660	0.6605
19	0.314	0.920	3.4346	1.620	2.794	
26				1.084	3.234	

¹Tissue homogenate concentration used was 50 mg/ml for brain and
25 mg/ml for heart.

²Absorbance at 535 mμ per mg PFA in heart or brain microsomal-
supernatant fraction.

The PFA level of the microsomal-supernatant fractions increases with age.

3. General Discussion

Adult rat brain contains relatively large amounts of PFA. The present study has shown that although brain and heart show different patterns of development, in the growing rat they exhibit similar trends insofar as the peroxidation of their polyunsaturated fatty acids is concerned. In both tissues the tendency of PFA to undergo peroxidation decreases progressively during early growth, reaching stable, minimum levels at about the 10th and 20th days in brain and heart respectively.

Even when the tissues were incubated in a well-oxygenated medium, the adult rat brain was less prone to produce peroxidation products, and contained a lower level of such materials than did brain tissue removed from the growing animal. Since the actual concentration of polyunsaturated fatty acids continually increases during growth, it is possible that some protective process, which protects the newly formed polyunsaturated fatty acids from oxidative breakdown, is introduced into or initiated in brain during its growth and development. Similar reasoning explains the behavior of heart polyunsaturated fatty acids.

Maturing brain may accumulate an antioxidant such as vitamin E. Some uncertainty concerning the function of vitamin E still exists. Many workers (136,137) are of the view that it functions principally as a general, natural antioxidant. It would be of interest to know its distribution in animal tissues, especially in the sub-cellular particles. It might then be possible to establish a relationship between the levels of vitamin E and of lipid peroxidation at the sub-cellular level. Work of this nature has been hindered by the lack of an adequate procedure for tissue work. Recently an extremely involved procedure has been applied for the estimation of vitamin E in animal tissues (138,139). However, using this method inconsistent results were obtained and it was concluded that the method would have to be further modified for tissue work.

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